

**PROTEIN PHOSPHORYLATION
IN MITOCHONDRIA
AND PLASMA MEMBRANES
FROM RAT LIVER**

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TABLE OF CONTENTS

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List of publications	4
Introduction	5
<u>Mitochondria</u>	
-General	10
-Protein kinases	11
-Choice of assay conditions	12
-Localization of protein kinases	13
-Identification of protein kinases	16
-Casein kinases	18
-Discussion	19
-Endogenous phosphorylation and phosphoprotein	22
-Choice of conditions for endogenous phosphorylation	23
-Localization of phosphoproteins	23
-Identity of the phosphoprotein	26
-The nature of the phosphorylated materiel of low molecular weight	27
-Discussion	29
<u>Plasma Membranes</u>	
-General	33
-Protein kinases and phosphoproteins	35
-Experimental conditions	36
-Protein kinases	37
-Endogenous phosphorylation and phosphoproteins	40
-Discussion	41
General discussion	45
Acknowledgements	48
References	49

LIST OF PUBLICATIONS

This thesis is based on the following publications which will be referred to by Roman numerals in the text.

- I. Protein Kinase Activity and Endogenous Phosphorylation in Subfractions of Rat Liver Mitochondria.
T. Henriksson and B. Jergil
Biochim. Biophys. Acta 588 (1979) 380-391.
- II. Localization of Protein Kinase Activity and Phosphoproteins in Mitoplasts from Rat Liver.
T. Henriksson and B. Jergil
Acta Chem. Scand. B 35, in press.
- III. Protein Kinase Activity and Endogenous Protein Phosphorylation in Rat Liver Plasma Membranes.
M. Sommarin, T. Henriksson and B. Jergil.
Acta Chem. Scand. B 35, (1981) 5-12.
- IV. Cyclic AMP-dependent Protein Phosphorylation on the Surface of Rat Hepatocytes.
M. Sommarin, T. Henriksson and B. Jergil
FEBS Letters 127 (1981) 285-289.

INTRODUCTION

Definition: Protein phosphorylation is the covalent attachment of a phosphate group to amino acid residues in a protein molecule.

Protein phosphorylation is well established in all living organisms from viruses (1) to mammals and plants (2). There are two basically different kinds of protein phosphorylation. The first kind is when a phosphate group becomes attached to the active site of an enzyme as a part of the catalytic cycle, while the second kind is when the catalytic cycle of the protein is not involved. The second kind of phosphorylation serves, in many cases, a regulatory function for the substrate protein. This thesis will consider only the second kind of phosphorylation.

Protein phosphorylation in liver occurs in all the major subcellular fractions, i.e. cytosol (3), mitochondria (4,1), endoplasmic reticulum (5,6), Golgi membranes (7), plasma membranes (5,7, III) and also ribosomes (8). Most of the proteins known to be regulated by phosphorylation-dephosphorylation are localized in the cytoplasm of the cell, e.g. phosphorylase kinase (9), glycogen phosphorylase (10,11), pyruvate kinase (12), phosphofructokinase (13), fructose-1,6-diphosphatase (14), and acetyl CoA carboxylase (15). Some noncytosolic proteins have also been shown to be regulated by a phosphorylation-dephosphorylation process. Two examples are pyruvate dehydrogenase

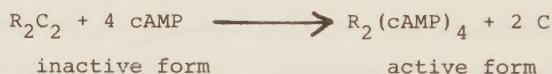
from mitochondria (16) and hydroxymethyl-glutaryl-CoA reductase from endoplasmic reticulum (17,18). It has also been shown that histones may undergo changes in their level of phosphorylation (19).

It can be noted that many of the phosphorylatable enzymes have a key role in the metabolism of the cell and are, therefore, ideal points of regulation. A particularly interesting feature is that several hormones act via protein phosphorylation-dephosphorylation mechanisms. The classical example is the regulation of glycogen metabolism outlined in fig. 1. The hormone in this case is glucagon. When this hormone binds to its receptor on the cell surface, the production of the so called second messenger' adenosine 3':5'-monophosphate (cAMP) will increase in the cell. This in turn activates a protein kinase, i.e. an enzyme that catalyzes the phosphorylation of proteins. This enzyme activates another protein kinase, phosphorylase kinase, via the phosphorylation of specific seryl residues of the protein. Phosphorylase kinase phosphorylates glycogen phosphorylase b (inactive form) to glycogen phosphorylase a (active form). The two kinases can also phosphorylate glycogen synthase I (active form) to glycogen synthase D (inactive form). This is a key mechanism for control of the blood glucose level. It should be stressed that this is a very simplified model and many regulatory mechanisms have been omitted for simplicity.

In order for a protein to be regulated by phosphorylation-dephosphorylation, the phosphorylated form must behave differently from the dephosphorylated form in a way significant

where N-P is the phosphate donor and P symbolizes a phosphate group or ion. The phosphate donor is usually ATP but may also be GTP (21), or in at least one case inorganic pyrophosphate (22).

The protein kinases have been classified into several subgroups with respect to their effectors. This classification is shown in Table 1. The cAMP dependent protein kinases consist of two regulatory and two catalytic subunits and they are regulated according to the following scheme (2):



where R indicates the regulatory and C the catalytic subunit.

Table 1

Classification of protein kinases according to Krebs and Beavo (2).

Category	Designation	Recognized entities
1	cAMP-dependent protein kinase	Type I and type II
2	cGMP-dependent protein kinase	One known entity
3	Ca ²⁺ -dependent protein kinase	Phosphorylase kinase and myosin light chain kinase ⁺
4	Double-stranded RNA-dependent protein kinase	One known entity
5	Nonspecified or messenger-independent protein kinases	Many known examples

⁺ Since this work was published several reports of other Ca²⁺-dependent protein kinases have appeared.

Type I and type II cAMP-dependent protein kinases have the same catalytic subunit but different regulatory subunits, and they behave differently with respect to temperature, salt and phosphorylation of the regulatory subunit (23-25). The cGMP-dependent protein kinase consists of two identical subunits which both have cGMP-binding and catalytic sites, and they do not dissociate upon binding of cGMP (26).

In rat liver the type I protein kinase has been found only in the cytosol, while the type II enzyme has been found also in particulate subfractions (27, I, III). A similar distribution of the two types of protein kinase has been found in cardiac muscle (28, 29).

The protein phosphatases have been much less studied although a couple of entities involved in glycogen metabolism have been isolated and characterized (30). They seem to have a rather broad substrate specificity and they are dependent on divalent cations. They are also regulated via specific protein inhibitors. The protein phosphatases will not be covered by this thesis, but the subject has been reviewed (see for instance ref 2, 30).

The purpose of this thesis has been to investigate the existence and location of protein kinases and phosphorylated proteins in mitochondria and plasma membranes from rat liver. A partial characterization of protein kinases has been performed and the identity of a mitochondrial phosphoprotein is proposed.

MITOCHONDRIAGeneral.

The mitochondrion consists of two different membranes, the outer and inner membranes, and two soluble fractions, the intermembrane fraction and the matrix. The two membrane fractions have very different properties. The outer membrane contains 50% lipid and is much more permeable than the inner membrane, which contains only 20% lipids. If the location of an enzyme within the mitochondrion is to be determined six different locations within the mitochondrion must be considered: The in- and outside of each membrane plus the two soluble fractions. Some enzymes occupy two of these locations by transversing the membrane. In order to study the inside-out topology of these membranes the outer membrane must be 'peeled off' leaving an intact mitoplast to be investigated or further subfractionated. Two methods have been tested during the course of this work to achieve this:

1. By partial solubilization of the outer membrane with digitonin (31).
2. By swelling the mitochondria in hypotonic phosphate buffer which will cause the breakage and detachment of the outer membrane (32).

The first method gave a low recovery of the mitoplast fraction (about 50% compared to the second method), and about 50% of the monoamine oxidase activity (marker for the outer membrane) was solubilized. The second method gave a higher contamination of outer membrane in the mitoplast fraction. Both methods gave a low recovery of

outer membranes as measured by marker enzymes. Since it was considered essential to work with intact membranes the solubilization of the outer membrane using the first method was unacceptable. The second method was therefore chosen for the experiments to be described.

The method used for subfractionation of mitoplasts (II) was modified from a method for extracting pyruvate dehydrogenase (16). This method gave a very good separation of membrane and matrix. The recovery of cytochrome oxidase (marker for inner membrane) was rather low (around 60%). But since the recovery of protein and protein kinase was around 80%, the rather low recovery of cytochrome oxidase was not considered to discredit the method.

Protein kinases.

The presence of protein kinases in rat liver mitochondria has been reported by various investigators (4, 5, 33-36). The number of protein kinases, their properties, and their intramitochondrial distribution has been less well characterized. It is also difficult to compare the results obtained by different research groups because of the different conditions used in their experiments. For instance, protein kinases have been extracted by mechanical means (36, 37), by salt (38, 39), by detergent (I, II), or by a combination of these conditions. The substrates used have been casein or phosvitin (33, 34, 36), histones (35, 36), endogenous heat-denaturated proteins (38), or protamine (I, II, 36, 38).

Choice of assay conditions.

Since we have been interested in the comparison of protein kinases present in different subfractions of the liver cell (7) we have tried to keep the experimental conditions as similar as possible between experiments with different subcellular fractions. The factor that seems to cause most difficulties when comparing results from different research groups is the choice of substrates. Protamine, that has been used here as an efficient substrate, has been found to be a less good substrate by other groups (36, 38). The reason for this is that the medium used by these groups for precipitating and washing the phosphorylated protamine, i.e. 10% trichloroacetic acid, results in a very low recovery. We have found that the recovery with this procedure is less than 10% due to the partial solubility of protamine in this medium. We have instead precipitated phosphorylated protamine with 5% trichloroacetic acid and 0.25% sodium tungstate, pH 2.1, (40) in which solution protamine is insoluble. Protamine then appears to be an excellent substrate (I, 27). The Mg^{2+} and ATP concentrations are not critical unless they become lower than 1 mM and 0.2 mM, respectively. High salt (0.3 M NaCl) is essential when protamine is used as a substrate since some protein kinases are dependent on salt for maximum activity with this substrate. The pH used is the same as has been used for other subfractions (7, 27), and is close to what other investigators use (34, 35, 41).

Localization of protein kinases.

The localization of protein kinases within mitochondria was performed in several steps. The first step was to fractionate mitochondria into outer membrane, intermembrane fraction and mitoplasts. As a second step inner membrane and matrix were separated. In this way it was possible to quantitate protein kinase in each subfraction. Since the membranes have two surfaces of which one is hidden from the assay mixture, the addition of detergent was necessary to express the full protein kinase activity of the membrane subfractions. One condition is that the protein kinase activity is unaffected by the detergent used. In experiments with the intermembrane fraction, that contains the same protein kinases as mitoplasts (I), there was no effect of 1% Triton X-100 on the protein kinase activity (42). Detergents can also be used to examine the location of protein kinases in a membrane. A latency in the enzyme activity indicates that the macromolecular substrate is not available for the enzyme which, therefore, may be hidden in the membrane or present on the unexposed side of the membrane. To confirm such experiments exposed protein kinases can be destroyed selectively by proteolytic enzymes before analysis of kinase activity in the presence and absence of detergent. Trypsin was used here since it had worked satisfactorily with other membranes (7, 27). The results obtained (II) and control experiments performed on the intermembrane fraction indicated that it worked properly in these experiments also.

The outer membrane fraction was subjected to trypsin and Triton X-100 treatment as was described for mitoplasts in (II). The results of these experiments (Fig.2) differed from those obtained with mitoplasts. The increase in protein kinase activity in presence of detergent was approximately 300%, about three times as much as in mitoplasts.

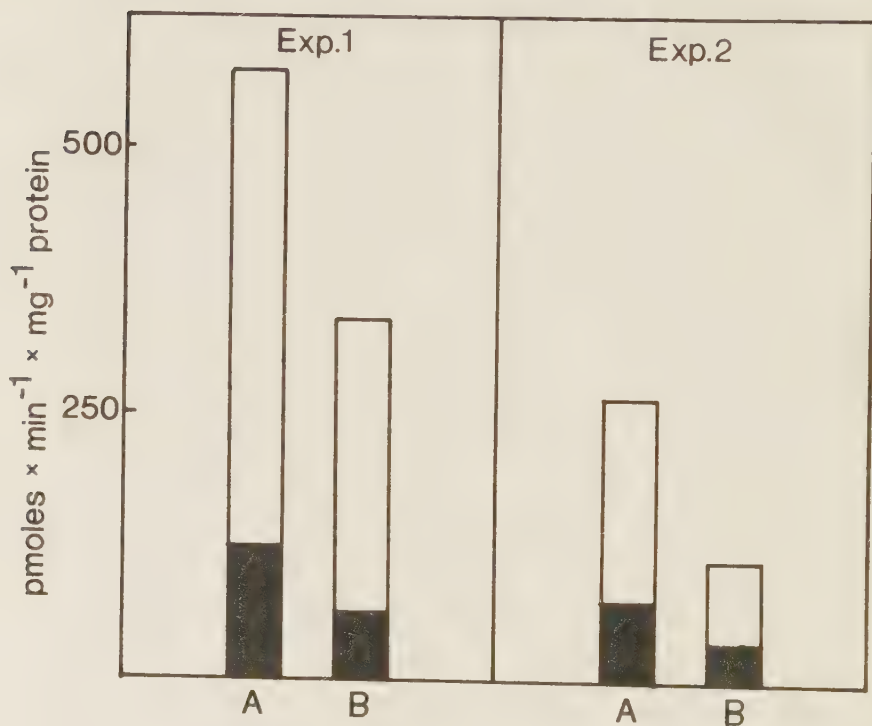


Fig. 2. Effect of trypsin treatment on the protein kinase activity of outer membranes.

Outer membranes were incubated with trypsin for 18 h at 0° C as described for mitoplasts in (II). Protein kinase assays were performed in the absence (closed bar) or presence (whole bar) of 1% Triton X-100. A, Untreated membranes; B, trypsin treated membranes. The figure shows the results of two experiments each including the combined material from three rats.

When outer membranes were treated with trypsin, the activity decreased by approximately 50% both when the assay was performed in the presence and absence of Triton X-100. This behaviour may be due to an activation of protein kinases in the outer membrane by detergent, an effect earlier shown with other protein kinases (43). With the data presently available it is not possible to determine the distribution of protein kinases across the outer membrane, so this membrane will be treated as one compartment.

From the results in (I) and (II) it is possible to calculate the amount of protein kinase present in the different locations of the mitochondrion (Table 2). Most of the protein kinase activity with protamine as substrate is localized in the intermembrane fraction, while the inner membrane and matrix have very low activities. The protein kinase activity recovered in the different subfractions has been corrected to allow for the low recovery of outer membranes and the contamination of outer membranes in the mitoplast fraction. The corrections are based on the recovery of marker enzymes. When calculating the distribution of protein kinase across the inner membrane both cross contamination of outer membranes and activation of protein kinase by Triton X-100 have been considered. The calculated distribution of protein in the mitochondrial compartments differs from that obtained by Schnaitman and Greenawalt (44). Inner membranes and the intermembrane fraction contains approximately 50% more, outer membranes 25% less and the matrix 50% less protein in my investigation.

Table 2.

Submitochondrial distribution of protein kinase.

<u>Location</u>	<u>Protein kinase activity (%)</u>
Outer membrane	18
Intermembrane fraction	72
Outside inner membrane	6.6
Inside inner membrane	1.5
Matrix	1.8

Identification of protein kinases.

The mitochondrial protein kinase activity could be separated into three fractions, eluting at 0.03, 0.10 and 0.20 M KCl, respectively, by DEAE-cellulose chromatography (I). These fractions have been designated MI, MII and MIII, respectively.

When these fractions were compared to protein kinases from the cytosol obtained with the same method (27) it was found that MI corresponded to the catalytic subunit of cAMP-dependent protein kinase (designated CI), MII corresponded to a cAMP-independent protein kinase (C I Ib) and MIII corresponded to a type II cAMP dependent protein kinase. No protein kinase corresponding to the type I cAMP-dependent protein kinase was found in mitochondria.

Microsomes contain three protein kinase fractions (27) corresponding to those found in mitochondria, although they are present in different proportions. Thus, the M II peak in mitochondria is much smaller than the corresponding peak in microsomes.

The isoelectric points of corresponding protein kinase fractions from the three different subcellular sources are very similar. This is a further indication that they are identical enzymes. The substrate specificity also indicates that corresponding protein kinase fractions are identical. This is supported by the fact that M I and M III are dependent on NaCl for maximum protein kinase activity when protamine is a substrate, while M II is independent of salt. The corresponding cytosolic fractions show the same behaviour. Since different protein kinases have different requirements for amino acid sequences around the residue to be phosphorylated (2), phosphorylation experiments with synthetic peptide substrates (so far available for cAMP-dependent protein kinases) and examination of phosphopeptides from various protein substrates should conclusively identify the protein kinases.

When protein kinase profiles of the intermembrane fraction and mitoplasts were compared to unfractionated mitochondria no significant differences were observed. Due to the small amount of material available and the lability of the enzymes chromatography profiles of protein kinases of outer membranes and subfractionated mitoplasts have not been obtained.

Table 3.

Comparison of protamine kinase activity and casein kinase activity in cytosol, mitochondria and mitochondrial sub-fractions.

The enzyme activities are expressed as $\text{pmoles} \times \text{min}^{-1} \times \text{mg}^{-1}$ protein. Each experiment included the combined material from three rats.

<u>Fraction</u>	<u>Protamine kinase</u>	<u>Casein kinase</u>
Experiment 1:		
Cytosol	1050	34.4
Mitochondria	21.2	3.0
Intermembrane fraction	168	8.7
Mitoplasts	15.7	0
Inner membrane	26.2	0
Matrix	14.2	0
Experiment 2:		
Cytosol	796	74.2
Mitochondria	15.4	2.2
Intermembrane fraction	206	15.4
Mitoplasts	6.3	0
Inner membrane	18.3	0
Matrix	7.4	0

Casein kinases

Several investigations have shown the presence of casein kinases in mitochondria (34, 36, 37, 41). In order to compare with our results mitochondria and mitochondrial subfractions

were assayed for casein kinase and protamine kinase activities. The assay mixture used for casein kinase analyses contained: 50 mM Tris-HCl pH 7.4, 5 mM MgCl_2 , 1 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (50 000 cpm/ nmole) and 2 mg/ml partially hydrolyzed casein (Sigma) (modified from (26)). The assay mixture was collected on filter paper discs and washed with 10% trichloroacetic acid and 1 M H_3PO_4 essentially as described in (IV). The background was obtained by omitting casein from the assay mixture. The incubation time was five minutes. Protamine kinase activity was analyzed as before. The results can be seen in Table 3. They show that the casein kinase activity is very low compared to protamine kinase activity (15% in whole mitochondria, 5% in intermembrane fraction, below detection limit in all other fractions examined). The specific activities of casein kinase obtained are comparable to those obtained by others (34, 45).

Discussion.

It has been shown here that mitochondria possess protein kinase activity that can be separated into three fractions by DEAE-cellulose chromatography. Of the substrates tested protamine is the most effective one. Most protein kinase activity is localized to the intermembrane fraction, while the amount of activity in the other submitochondrial fractions is low. The correlation between our results and those of other investigators will be discussed below.

Sankaran et al. (34, 37) have investigated protein kinases from liver mitochondria of normal and tumour-bearing rats.

The extracted protein kinase with sonication which was followed by ammonium sulphate fractionation. They then separated a histone kinase and a casein kinase by DEAE-cellulose chromatography. Considering the salt concentration where the kinases elute, their kinase I' (34) might correspond to our M III. The reason why they did not find additional protein kinases corresponding to our M I and M II might be that they were lost during the prepurification procedure, or that they eluted in the void volume since they started their chromatography at a higher salt concentration. We have not found any protamine kinase activity eluting at higher salt concentrations than M III and, therefore, we cannot confirm the existence of kinase II'. They also stated that neither of the protein kinases phosphorylated protamine (34). The reason for this is the improper washing procedure used in their analysis.

Kleitke et al. (35, 46) have found protein kinase activity that acts on heat-treated mitochondrial proteins and histones, but not on protamine and casein (46). With the exception of the lack of activity with protamine (which is due to the improper precipitation procedure above) these results are in accordance with ours. They also found about 100% increase in protein kinase activity upon treatment with 1% Triton X-100. This treatment also released cAMP-dependent protein kinase activity not found in untreated mitochondria. In (I) it was found that whole mitochondria have cAMP-stimulated protein kinase activity. A possible explanation for the difference is that Kleitke et al. removed the intermembrane fraction, where most of the cAMP-stimulated protein kinase activity

is located (I), by their harsh treatment of the mitochondria. We used bovine serum albumin to protect and stabilize the mitochondria during the preparation.

The DEAE-cellulose chromatography profile presented (35) resembles the one found here (I) with essentially three protein kinase peaks. They found that the second peak was cAMP-stimulated instead of the third one found here. The reason might be that the two different species used (guinea pig and rat, respectively) have functionnally very similar protein kinases that have different chromatographic behaviour.

Vardanis (36) has investigated protein kinase activity in mouse liver and mouse heart mitochondria using a histone mixture as a substrate. He found that the protein kinase activity was associated with the inner membrane. Since no data on the intermembrane fraction marker enzyme was presented, it is not possible to deduce the fate of the intermembrane fraction, where most of the protein kinase activity is located (I). Vardanis also found that large amounts of protein kinase activity was released upon sonication or freezing and thawing (8-10 times the original activity). Neither we nor Kleitke et al. (personal communication) have been able to reproduce these results. Vardanis also reported a very low protamine kinase activity due to the improper analytical procedure discussed earlier.

From what has been discussed here the following conclusions can be drawn:

1. Rat liver mitochondria contain a protein kinase activity that is mainly localized to the intermembrane fraction.
2. This activity can be separated into three fractions by ion-exchange chromatography, and these fractions are most likely identical to protein kinases obtained from the cytosol and microsomes.
3. The identity of these three fractions is probably: M I, the catalytic subunit of cAMP-dependent protein kinase; M II, a cAMP-independent protein kinase identical to C I Ib; M III, a type II cAMP-dependent protein kinase.
4. The exogenous substrate tested was protamine. There exists a protein kinase activity active on casein in mitochondria, but this is low and might be effected by the same enzymes responsible for protamine phosphorylation.

Endogenous phosphorylation and phosphoproteins.

Up to now only two mitochondrial proteins have been shown to be regulated by phosphorylation-dephosphorylation, namely pyruvate dehydrogenase (16) and branched-chain 2-oxoacid dehydrogenase (47). The first enzyme becomes phosphorylated on three different seryl residues in the α -subunit (48), but only one of these has a regulatory function. The site of phosphorylation on the second enzyme is on its α -subunit (47) although the number and identity of the amino-acid residues to be phosphorylated has not yet been determined. In both cases the phosphorylated enzyme is the inactive form. Pyruvate dehydrogenase exists in the matrix.

Succinyl CoA-synthetase also becomes phosphorylated (49) but in this case the phosphorylation is a part of the catalytic cycle of the enzyme. The site of phosphorylation is also different, in this case it is a histidyl residue. This enzyme is located on the inner membrane of the mitochondrion (50).

Choice of conditions for endogenous phosphorylation.

The endogenous incorporation of phosphate into mitochondria shows a broad pH-optimum from pH 6.5 to 8.5 (42). The Mg^{2+} and ATP concentrations are not critical as long as they are kept between 1 mM and 20 mM (for Mg^{2+}) and 0.2 mM and 2 mM (for ATP).

Localization of phosphoproteins.

When mitochondria or mitochondrial subfractions was incubated with $[\gamma\text{-}^{32}\text{P}]$ ATP most incorporation of phosphate into TCA-tungstate precipitable material was in the mitoplast fraction (I). Although the total incorporation into outer membranes was low the specific incorporation was high. In contrast there was no incorporation of phosphate into the intermembrane fraction.

Analysis of the phosphorylated material by SDS-polyacrylamide gel electrophoresis (I) showed that the mitoplast fraction contained a double phosphoprotein band at M_r 47 500, and that the incorporation in outer membranes was into material of comparatively low molecular weight that moved just behind the salt front.

There were no phosphoprotein bands in the intermembrane fraction. When mitoplasts were subfractionated into inner membrane and matrix, followed by phosphorylation and gel electrophoresis (II), the phosphoprotein band was distributed between the two fractions so that two thirds followed the matrix and one third the inner membrane. In comparison, the distribution of the matrix marker enzyme glutamate dehydrogenase was 90% in the matrix fraction and 10% in inner membranes. This indicates that the phosphoprotein tends to associate with the inner membrane.

In order to confirm the matrix location of the phosphoprotein band intact mitoplasts were treated with trypsin in the presence and absence of Triton X-100. This experiment showed that the [³²P] incorporation was sensitive to trypsin treatment both in the presence and absence of Triton X-100, although the incorporation was much less affected in the absence of detergent. Isolated matrix which had been trypsin-treated in the absence of detergent did not incorporate [³²P] at all.

An explanation for the inhibition of phosphate incorporation into mitoplasts after trypsin treatment in the absence of detergent might be that the influx of ATP (51) into mitoplasts decreased by 50-70% (II). The amount of ATP within the mitoplasts may therefore become limiting.

When isolated outer membranes were phosphorylated and examined by SDS-polyacrylamidegel electrophoresis the in-

corporation of phosphate was into material of comparatively low molecular weight. However, when the outer membranes were treated with Triton X-100 before endogenous phosphorylation four phosphopeptides (M_r 92 000, 77 000, 61 000 and 57 000) were resolved by electrophoresis (Fig. 3). In addition, a highly increased phosphorylation of the low molecular weight material was observed.

There are several possible reasons why these proteins become phosphorylated in the presence of Triton X-100. For instance it is possible that protein kinase and its substrate cannot interact in the intact membrane. Other possible

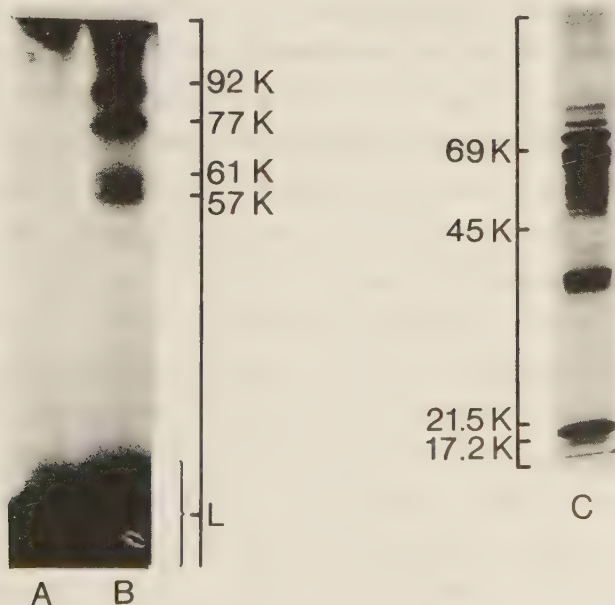


Fig. 3. Latency of phosphoproteins in outer mitochondrial membrane.

Outer mitochondrial membranes were incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, and subjected to SDS-polyacrylamidegel electrophoresis and autoradiography as described in (1). A, untreated membranes; B, Triton X-100-treated membranes and C, protein pattern.

explanations are that Triton X-100 activates protein kinases, or that the detergent changes the conformation of substrate proteins to make them susceptible to phosphorylation. Since, in intact mitochondria, ATP can pass through the outer and inner membranes to phosphorylate matrix proteins, the explanation for the latency of these phosphoproteins cannot be that the membrane prevents ATP from reaching the protein kinases. Whether or not these proteins are phosphorylated in the intact cell is still an open question.

Identity of the phosphoprotein.

The identity of the phosphoprotein is still unclear although pyruvate dehydrogenase seems to be the most likely candidate. Morgan and Routtenberg have shown (52) that a phosphoprotein doublet band in rat brain mitochondria is pyruvate dehydrogenase. This doublet had a M_r of 41 000 and comigrated with the α -subunit of purified phosphorylated pyruvate dehydrogenase from bovine kidney. The latter also appeared as a doublet. The phosphorylation of the brain protein band was strongly inhibited by 5 mM pyruvate, 5 mM dichloroacetic acid, or 1 mM NAD, known inhibitors of pyruvate dehydrogenase kinase (53-55). The phosphorylation of the rat mitochondrial protein was also inhibited strongly by 5 mM pyruvate, 5 mM dichloroacetate and 1 mM NAD (II), and the degree of inhibition was similar to that presented in (52). The rather high M_r (45 000 - 47 500) determined in (I,II) might be due to the use of different molecular weight markers. Although branched chain 2-oxoacid dehydrogenase (BCDH) complex from rat liver recently has been shown to be

phosphorylated (56) and its M_r (48 000) is closer than that of pyruvate dehydrogenase to the one reported here, its reported behaviour (56) is quite different from the behaviour of the phosphoprotein band found in our experiments. For instance the phosphorylation of the BCDH complex in rat liver has a 10 min lag period and it is not inhibited by dichloroacetate ion. The identity of the phosphoprotein may be settled when purified pyruvate dehydrogenase from rat liver mitochondria is available to us. For several reasons it is much less likely that the phosphorylated protein is succinyl CoA-synthetase. For instance, the M_r of its phosphorylated subunits is 34 500 (49), it is bound to the inner membrane (51) and it is phosphorylated on a histidine residue. All of these facts disagree with the behaviour of the phosphoprotein band.

The nature of the phosphorylated material of low molecular weight.

The incorporation of phosphate into the outer mitochondrial membrane is nearly entirely into low molecular weight material. It has been shown that $[^{32}\text{P}]$ is incorporated into phosphatidylinositol upon incubation of mouse liver microsomes with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (57). The extraction properties of phosphoinositides differ from other phospholipids. They do not extract readily with ethanol:ether (1:1) or chloroform:methanol (2:1) as do other phospholipids. This means that they might remain in the filter paper discs during the washing procedure used in the protein kinase assays (I).

In order to examine whether phosphate was incorporated into phosphatidylinositol in the outer membrane fraction this was incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ for 5 min as described in (I). The membranes were precipitated and washed twice with 10% trichloroacetic acid. The precipitate was then extracted twice with 1 ml of chloroform:methanol:conc. HCl (200:100:1, by volume). The extract was evaporated under N_2 and the residue dissolved in a small volume of chloroform. The phospholipids extracted in this manner were subjected to thin layer chromatography as described in (57). The autoradiography from the chromatogram (Fig. 4) showed that approximately 90% of the radioactivity was recovered in a spot coincident with authentic 4'-monophospho phosphatidylinositol. There was also a small amount of a spot coincident with 4', 5'-diphospho phosphatidylinositol and of two unknown substances. In mouse liver microsomes (57) only monophospho phosphatidylinositol was found.

Phosphatidylinositol has been shown to undergo phosphorylation in all major membrane fractions in the cell (58). The phosphorylation of phosphatidylinositol in microsomes, lysosomes and mitochondria was enhanced by the non-ionic detergent Cutscum. It appears from our results that phosphorylation of phosphatidylinositol in mitochondria occurs mainly in the outer membrane and that the reaction is enhanced by Triton X-100.

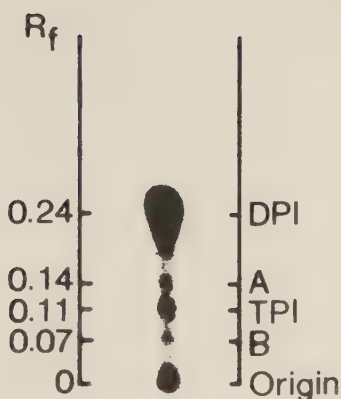


Fig. 4. Thin-layer chromatography of phosphoinositides from mitochondrial outer membranes.

Outer membrane fraction was incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Phosphoinositides were extracted and chromatographed as described in the text. The inositides were identified by co-chromatography of reference substances obtained from Sigma. The R_f values are indicated on the left side of the chromatogram. The right side identifies the substances: DPI, 4'-monophospho phosphatidylinositol; TPI, 4', 5'-diphospho phosphatidylinositol; A and B are unidentified substances.

Discussion

One interesting observation is that protein kinase activity and phosphorylatable proteins have completely different distributions in rat liver mitochondria. Thus, most of the protein kinase activity is present in the inter-membrane fraction where no protein substrates can be detected.

The major phosphoprotein is instead located in the matrix which is low in protein kinase activity. In addition this phosphoprotein is likely to be pyruvate dehydrogenase, which is not supposed to be a substrate of the protein kinases studied here. One possibility is that the protein kinase activity found in mitochondria is of cytosolic origin. This does not seem to be the case, however, since the relative specific activity of protein kinase in mitochondria is much higher than that of the cytosolic marker enzyme lactate dehydrogenase. Furthermore the type I cAMP-dependent protein kinase that is abundant in the cytosol is absent from mitochondria. It appears also that the protein kinase activity of the intermembrane fraction is partly masked in intact mitochondria since the recovery is approximately 300% upon extraction.

An explanation to the apparent lack of substrates for the mitochondrial protein kinases could be that the phosphorylation in vivo is different from that obtained in vitro. This has been shown to be the case for the cytosol (59) where 3 times as many proteins become phosphorylated when hepatocytes are incubated with $[^{32}\text{P}]$ -phosphate than when isolated cytosol is incubated with $[\gamma\text{-}^{32}\text{P}]$ ATP. Other less likely explanations are that phosphorylatable proteins become proteolyzed during the preparation, that there are too low amounts of phosphoproteins to be detected by the methods used, or that the phosphorylatable proteins are already phosphorylated in isolated mitochondria.

In preliminary experiments hepatocytes were incubated with $[^{32}\text{P}]$ -phosphate and subfractionated. The fraction containing mitochondria (320 x g pellet) showed several phosphoprotein bands on electrophoresis and autoradiography. In contrast, when the same fraction was phosphorylated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ after subfractionation only one phosphoprotein band with M_r 47 500 was seen (43). Although the 320 x g pellet also contained nuclei, this experiment indicates that the phosphorylation in the intact cell is different from that of isolated subcellular fractions.

Siess and Wieland (59) have presented a phosphoprotein pattern of mitochondria from hepatocytes incubated with $[^{32}\text{P-}]$ -phosphate. This resembled the pattern obtained in the 640 x g - 6900 x g pellet. This fraction contained considerable amounts of plasma membranes as judged by the content of 5'-nucleotidase (marker enzyme for plasma membrane) (42). Most phosphoproteins in this fraction appear to be derived from the plasma membrane since the phosphoprotein pattern resembled the pattern obtained from purified plasma membranes (III).

The results from the mitochondria can be summarized in the following points:

1. The mitochondria have two protein kinases that can use protamine as substrate, one cAMP dependent and one cAMP independent. These protein kinases also occur in microsomes and cytosol. The main location for protein kinases in mitochondria is in the intermembrane fraction.

2. The mitochondrial substrates in vivo for these protein kinases are still unknown and do not function as substrates in the in vitro assay. Preliminary experiments with hepatocytes indicates their possible existence.
3. The main phosphorylatable protein in mitochondria is pyruvate dehydrogenase. It has been shown in other species (16) to be phosphorylated by a specific protein kinase. This enzyme complex exists in the matrix.
4. Phosphatidylinositol is phosphorylated to monophospho- and diphospho- phosphatidylinositol in the outer membrane. The function of this phosphorylation is not known.

PLASMA MEMBRANES

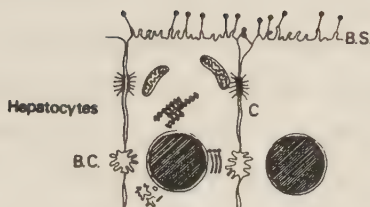


Fig. 5. Schematic drawing of hepatocytes showing the major domains of the plasma membrane (60). The domains indicated are: B.C., bile canaliculi; B.S., blood sinusoidal; and C., contiguous membrane.

General.

The plasma membrane is laterally differentiated into three major specialized areas or domains with different functions. These are: the blood sinusoidal membrane which is the main site for transport of metabolites and for binding of hormones; the contiguous membrane, where cell-cell adhesion and cell-cell communication takes place; and bile canaliculi, where the bile is excreted (60). Both the sinusoidal and the canalicular membranes have their surface areas increased by microvilli.

Plasma membranes, being a site of hormone receptors, is an interesting organelle from a regulatory point of view and the phosphorylation processes, being an important regulatory mechanism, should be investigated. Up to now, no other research group has characterized protein kinases or phosphoproteins in rat liver plasma membranes. In order to do this, isolated liver plasma membranes were examined for the presence of protein

kinase activity directed towards exogenous (protamine and histone) or endogenous substrates. The protein kinase activity was extracted from the membrane, purified and further characterized. The phosphoproteins of the membrane were characterized by their apparent molecular weight on SDS-polyacrylamidegel electrophoresis. Furthermore, protein kinase activity and phosphorylatable proteins was shown for the first time to be present on the outer surface of isolated hepatocytes. The purpose of these studies was to establish a basis for further investigations on the function and identity of protein kinases and phosphorylatable proteins in plasma membranes.

Liver plasma membranes were prepared according to Aronson and Touster (61). They were obtained in two fractions designated N_2 and P_2 . The N_2 fraction was recovered together with the nuclei, while the P_2 fraction was obtained from the microsomal fraction. Since the liver plasma membranes have specialized domains (fig. 5) it could be expected that N_2 and P_2 are derived from different regions of the cell surface. According to Evans (60) the P_2 fraction originates in the blood sinusoidal membranes, while the N_2 fraction contains both contiguous and canalicular material. The latter fraction was the only one that was purified enough to give significant results and was chosen for further experiments. The P_2 fraction was rather contaminated by intracellular organelles, especially Golgi membranes. Although the structure of the isolated N_2 fraction was reported to be lamellar sheets (60) we have electron microscopical evidence that the fraction predominantly consists of vesicles (62). The discrepancy could

be due to repeated ultracentrifugation and homogenization of the isolated fraction in our experiments.

Hepatocytes were prepared according to a standard procedure (63) by perfusion of the liver with a solution containing collagenase. To enhance the viability of the cells (as measured as the exclusion of 0.1% Trypan Blue) 0.1% hyaluronidase and 0.2% bovine serum albumin were included in the perfusion medium.

Protein kinases and phosphoproteins.

Phosphorylation of proteins in plasma membranes has been shown to occur in several types of cells, e.g. in erythrocytes (19), nerve cells (19), fibroblasts (64) and ovary cells (65). In liver cells incorporation of ^{32}P into plasma membrane proteins has been shown by Blat and Harel (66, 67), but no further characterization of the proteins involved was done. In 1971 Shlatz and Marinetti (68) found that rat liver plasma membrane proteins could be phosphorylated by cytosolic protein kinases in a cAMP-dependent manner, and in 1979 Carstens and Weller (69) found endogenous cAMP-dependent phosphorylation of proteins in plasma membranes from rat liver. In none of these cases has there been any characterization of the protein kinases or protein substrates involved. Recently, Marchmont and Houslay (70, 71) provided evidence that one type of cAMP-phosphodiesterase is regulated by phosphorylation and that this is dependent on insulin and cAMP.

During the last years protein kinase activity has been shown to be present on the outer surface of several types of cells, e.g. on adipocytes (72), fibroblasts (73) and spermatozoa (74). On adipocytes and spermatozoa the protein kinase activity was stimulated by cAMP. Malignant fibroblasts had cAMP-dependent protein kinase activity, while the activity on normal fibroblasts was not affected by this nucleotide (73). The report on adipocytes (72) also shows that substrates for protein kinase are present on the outside of the cell.

It can be seen from what is said above that there is a need for a study of the protein kinases and phosphorylatable proteins in liver plasma membranes, with a characterization and identification of the proteins involved.

Experimental conditions.

In order to measure protein kinase activity and endogenous incorporation of [32 P]-phosphate into isolated plasma membranes the same methods as earlier were used with the following modifications: the ATP concentration was increased to 2 mM and the ATP to protein ratio was kept at 33 μ moles ATP/mg protein for the protein kinase assay and 5 μ moles ATP/mg protein when endogenous incorporation of phosphate was measured (III). In the latter measurements 20 mM NaN_3 was also added to the assay mixture. These measures were undertaken to compensate for the hydrolysis of ATP by ATPases present in the plasma membrane. Fig. 1. in paper III shows that these modifications generate a longer linear reaction in the protein kinase assay without affecting the initial reaction rate. Control experiments also showed that these modifications did not affect the

relative intensity of the phosphoprotein bands obtained after SDS-polyacrylamidegel electrophoresis and autoradiography; they only affected the total [^{32}P]-incorporation to plasma membrane proteins.

When protein kinase activity and endogenous incorporation of phosphate to proteins in isolated hepatocytes was measured, the assay medium used was Hanks' Ca^{2+} -free medium (this was also used during the isolation of the cells). The ATP concentration was 2 mM, the same as for isolated plasma membranes, and the incubation temperature was 37°C . The substrate in the protein kinase assay was a histone mixture (Sigma type II-A) instead of protamine since the latter caused aggregation and destruction of the cells. In order to confirm the surface location of the protein kinase activity, hepatocytes were treated with hydroxyapatite-agarose gel, during the preparation. This gel will bind soluble protein kinases that leaks out into the medium. Other control experiments showed that the histones did not induce significant leakage of protein kinases from the cells into the medium during the short incubations performed.

Protein kinases.

As can be seen in (III) plasma membranes contain a tightly bound protein kinase activity that is not activated by cAMP. This activity can be solubilized with 1M KCl and 1% Triton X-100 and then separated into two (or occasionally three) fractions (designated PM I and PM II) by DEAE cellulose chromatography. Some properties of these enzymes and other protein kinases obtained from cytosol, microsomes and mitochondria

Table 4

Comparison of protein kinases from different subcellular fractions.

Protein kinase	Elution point M KCl	Isoelectric point		Substrate specificity			
		-cAMP	+ cAMP	Protamine +NaCl	-NaCl	PKInh +NaCl	Histone
Peak I							
Cytosol	0.036	8.10	8.15	100	41	24	7
Microsomes	0.035	8.05	8.05	100	46	-	-
Mitochondria	0.032	8.05	8.15	100	39	14	15
Plasma membr.	0.038	8.10	8.10	100	42	14	18
Peak II							
Cytosol	0.11	5.45	5.45	100	90	97	4
Microsomes	0.10	5.50	5.50	100	114	-	-
Mitochondria	0.10	5.45	5.45	100	107	-	11
Plasma membr.	0.22	(5.5) (4.9)	-	100	106	70	9
Peak III							
Cytosol	0.20	4.90	8.15	100	77	52	12
Microsomes	0.20	4.80	8.10	100	62	-	-
Mitochondria	0.20	4.75	8.20	100	45	-	9
Plasma membr.	nonexistent						

Since protein kinase corresponding to peak IIa in the cytosol (27) can not be detected in any of the particulate fractions this has been excluded from the table. The substrate specificity was measured in the presence of 5 mM cAMP. PKInh is protein kinase inhibitor. The data in this table are from (27, I, III).

have been compared in table 4. Peak I from all four subfractions appears to be identical constituting the catalytic subunit of cAMP-dependent protein kinases (27). Peak II(b) from mitochondria, microsomes and cytosol have similar properties as peak III from the same sources. These peaks most likely constitute a cAMP-independent protein kinase not yet described in the literature and the type II cAMP-dependent protein kinase, respectively. The identity of peak II from plasma membranes is less certain. It is likely to be a mixture of the peak II(b) and peak III protein kinases existing in cytosol, microsomes and mitochondria since a) inhibition by protein kinase inhibitor

is the average of what is obtained from peak II and peak III and b) isoelectric focusing, although not conclusive, shows two protein kinases with the same isoelectric points as peak II and III from the other subfractions mentioned, respectively. There is also a possibility that PM II is a protein kinase specific for plasma membranes.

When intact hepatocytes were investigated (IV) protein kinase activity was found on the outside of the cells. Unlike the activity in isolated plasma membranes the activity on hepatocytes was stimulated by approximately 50% by cAMP. This stimulation, but not the basic protein kinase activity, was unstable and was not observed after a couple of hours. To prove the outside location of the protein kinase activity the cells were treated with hydroxyapatite-agarose gel during the preparation procedure. This treatment was performed to adsorb protein kinase which might be released during the preparation, and then bound inadvertently on the outside of the cells. Although the protein kinase activity in the medium decreased by 80% in the presence of hydroxyapatite the cell surface activity was not affected (IV). This suggests a surface location of protein kinase on hepatocytes. The question whether there is protein kinase attached to the cytosolic side of membranes is less clear. The protein kinase activity in these membranes did not show any latency upon treatment of the membranes with Triton X-100 (III). This is not necessarily a criterium for the same transverse orientation of all protein kinases. For instance, the membrane vesicles might not be completely closed. The fact that cAMP-phosphodiesterase

can be phosphorylated by endogenous enzymes (70) indicates that there is protein kinase associated with the cytosolic side of the membrane.

Endogenous phosphorylation and phosphoproteins

After incubation of isolated plasma membranes with $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$, six major phosphorylated components with M_r 104 000, 84 000, 56 000, 51 000, 21 000 and 18 000 and several minor ones could be detected after SDS-polyacrylamidegel electrophoresis and autoradiography.

When hepatocytes were incubated under similar conditions several protein bands became phosphorylated and the phosphorylation of five of these was activated by cAMP. The M_r of the cAMP-dependent phosphoprotein bands were: 105 000, 84 000, 75 000, 60 000 and 52 000. It appears from these results that M_r 105 000, 84 000 and 52 000 components from isolated plasma membranes and hepatocytes may be the same. In addition the M_r 57 000 component, whose phosphorylation is not stimulated by cAMP, occurs both in hepatocytes and isolated plasma membranes. The M_r 21 000 and 18 000 components of isolated plasma membranes can also be seen, although weakly, in hepatocytes. The lack of cAMP-dependency of the M_r 105 000, 84 000 and 52 000 components in isolated plasma membranes is probably due to the lability of this stimulation, which was mentioned earlier.

When isolated plasma membranes were extracted with 1 M KCl (III) four additional phosphoprotein bands appeared in the extract. These had M_r 59 000, 49 000, 41 000 and 39 000. The

residue after extraction retained the same phosphoprotein pattern as untreated plasma membranes. After extraction in 1 M KCl and 1% Triton X-100 only two of these components (M_r 59 000 and 41 000) were found in the extract. In addition, the M_r 104 000 and 51 000 components present in untreated plasma membranes were found both in the extract and the residue after extraction. The M_r 18 000 component was found neither in the extract nor in the residues.

The question of the transverse orientation of the phosphoproteins in the plasma membrane is still open although some conclusions can be drawn. The major phosphorylated components found on hepatocytes are obviously exposed on the outer surface of the cell. The M_r 21 000 and 18 000 components, being very weakly phosphorylated in hepatocytes, could be on the cytoplasmic side of the plasma membrane, although this is not certain. The reason for the extra phosphoprotein bands to appear upon extraction of isolated plasma membranes could be due to restrictions in the enzyme-substrate interactions in the native membrane, i.e. that the enzyme and its substrate are either located on different sides of the membrane or that the lateral movement of these is restricted.

Discussion

It has been shown here that plasma membranes contain both protein kinases and phosphoproteins that are tightly bound to the membrane. The protein kinase activity could be separated into two fractions, one of which was identified as the catalytic subunit of the cAMP-dependent protein kinases, the other

preliminarily identified as a mixture of a cAMP-independent protein kinase specific towards basic proteins, and the type II cAMP-dependent protein kinase. The latter two enzymes have been found in all subfractions of the liver cell investigated by us so far (27, I, III), while the type I cAMP-dependent protein kinase has been found only in the cytosol (27). Furthermore, the cAMP-independent and the type II enzyme but not the type I enzyme from cytosol will bind to microsomal membranes in vitro (27). This could indicate that the type II enzyme is mainly phosphorylating membrane proteins while the type I enzyme is acting only on cytosolic proteins. Another possibility is that the type II enzyme is regulated in two ways: by binding to different membranes and by cAMP-activation. The findings of Shlatz and Marinetti (68) that cytosolic protein kinases can phosphorylate plasma membranes in a cAMP-dependent way supports this hypothesis. Since the difference between the type I and type II enzymes lies in the different regulatory subunits, it seems likely that the regulatory subunit determines the binding to the membranes. It has been shown on spermatozoa (74) that low amounts ($2.5 \mu\text{M}$) of cAMP will release protein kinase activity previously bound to the membranes. In liver membranes the situation seems to be different. All membrane fractions examined have substantial amounts of free catalytic subunit (27, I, III), and treatment of hepatocytes with cAMP did not cause a significant release of protein kinase activity. The reason could be that the catalytic subunit remains bound to the membrane when membrane-bound holoenzyme dissociates. This hypothesis is summarized in Fig. 6.

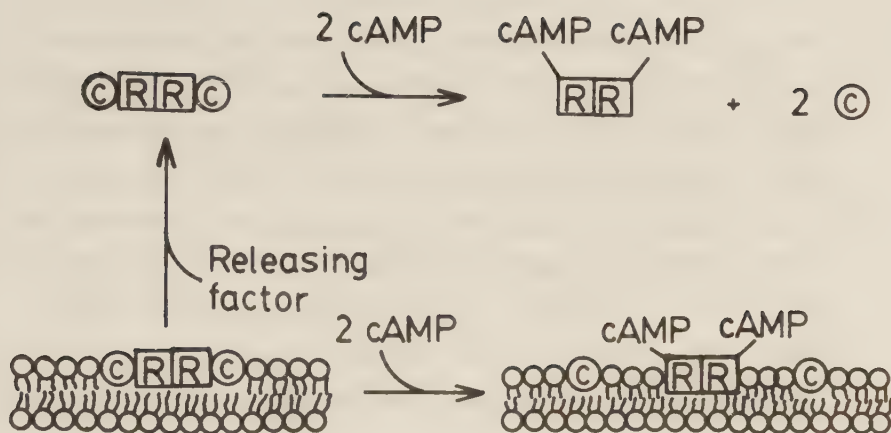


Fig. 6. Hypothesis for the regulation of type II cAMP-dependent protein kinase.

The transverse location of protein kinases in the plasma membrane is still an open question. It has been shown here (IV) that they exist on the outside of the liver cell, and there are indications (70) that they might be present on the cytosolic side as well. The lack of latency of protein kinase activity upon treatment of plasma membranes with Triton X-100 is not an evidence for the absence of protein kinase activity on the cytosolic side of the membrane. There are alternative explanations; the plasma membrane vesicles used here might not be completely sealed; or protein kinases existing on the cytosolic side might not use basic proteins as substrates.

Marchmont and Houslay (71) have shown the phosphorylation of five proteins in rat liver plasma membranes. Two of these (M_r 140 000 and 80 000) were dependent on μM concentrations of cAMP for phosphorylation while the three others (M_r 52 000, 28 000 and 14 000) were dependent on both insulin and cAMP.

The M_r 52 000 component was identified as cAMP-phosphodiesterase . There could be several reasons for the discrepancy between these results and ours. The preparation procedure for plasma membranes used by Marchmont and Houslay (75) gives only 50% of the relative specific activity of 5'-nucleotidase (marker enzyme for plasma membranes) compared to our preparation. On the other hand it is reported to take only three hours (75). They also used a substantially lower ATP to protein ratio than we did and without addition of any ATPase inhibitor.

One important factor in determining the functions of phosphoproteins is their transverse location. Since ATP most likely is an intracellular molecule, only proteins on the cytosolic side of the membrane should become phosphorylated except under special circumstances. One possible function for external phosphorylatable proteins could be to trigger cell division caused by damage to other cells. In this case cytosol containing ATP leaks into the intercellular space, where it can be used to phosphorylate external phosphorylatable proteins. This phosphorylation could for instance in turn activate the production of a second messenger inside the cell which subsequently could trigger cell division.

Phosphoproteins on the cytosolic side should have functions that are more part of the "everyday life" of the cell. Such functions have been suggested in other types of cells. Spectrin (a phosphoprotein in the erythrocyte membrane), for instance, is supposed to interact with the cytoskeleton, and this interaction might be regulated by phosphorylation-dephos-

phorylation. It has also been suggested that phosphorylation-dephosphorylation processes regulate the membrane permeability in nerve cells (77, 78) and erythrocytes (79). In kidney plasma membranes there is evidence for phosphorylation of Na^+ , K^+ -ATPase by protein kinase (80). Similar functions for phosphoproteins in liver plasma membranes are possible, although these questions can not be settled until the phosphoproteins have been identified.

GENERAL DISCUSSION

The most investigated regulatory function of protein phosphorylation is as a part of the hormonal control process, especially the hormones glucagon, catecholamines and insulin. All these act through second messengers although the second messenger of insulin (81) is still not identified. These hormones regulate the metabolism in liver in a way where insulin essentially opposes the action of the other hormones.

Glucagon acts by increasing the cAMP level in the cell thereby activating cAMP-dependent protein kinases as described in the introduction. Insulin reverses the increase in cAMP caused by glucagon by phosphorylation and activation of cAMP-phosphodiesterase (70), but it does not affect the basic cAMP level. Insulin also causes the production of at least one second messenger with specific metabolic effects, i.e. activation of pyruvate dehydrogenase through activation of pyruvate dehydrogenase phosphatase (82) and activation of protein kinases (83).

The roles of membrane-bound protein kinases and phosphorylatable proteins are in most cases completely unknown. In some instances protein phosphorylation in membranes is part of the normal metabolic regulation. An example of this is hydroxymethyl-glutaryl CoA reductase (16, 17), a key enzyme in the synthetic pathway for cholesterol, that is deactivated through enzymatic phosphorylation when the cell is exposed to glucagon and reactivated by insulin. The phosphorylation of cAMP-phosphodiesterase could have a different function, by enhancing the switch-over of the cell metabolism from an essentially glucagon or catecholamine controlled state to an insulin controlled state. There is also the possibility that other phosphoproteins cause the reverse effect by inactivating the second messenger of insulin.

In the plasma membrane section a hypothesis on the function of membrane-bound type II cAMP-dependent protein kinase was discussed. The distribution of this enzyme within the cell could determine the phosphorylation reactions of different metabolic pathways. For instance, the soluble protein kinase is likely to act on the glycolytic-gluconeogenetic pathways, while the enzyme when bound to microsomes can regulate the biosynthesis of cholesterol through phosphorylation of hydroxymethyl-glutaryl CoA-reductase.

How the cell regulates the binding of protein kinases to membranes is another interesting question. A specific membrane receptor protein is one possibility. Phosphorylation of the

regulatory subunit, that is already known to affect the stimulation by cAMP (2), could also affect the binding to membranes. A third possibility is that the phosphorylation of phosphatidylinositol can be involved. This phosphorylation can be regulated by hormones (4), and the introduction of extra negative charges in the membrane might affect the binding of proteins. It is also possible that there is more than one mechanism involved. The reason for binding of protein kinases to membranes would of course be economy for the cell, that would only need to produce one enzyme where it otherwise would have to produce several ones.

The liver cell is quite complex with many different functions, and it needs many regulatory mechanisms to function properly. The phosphorylation-dephosphorylation process has shown itself to be both basically simple and very versatile, and it is therefore not surprising to find many phosphorylatable proteins in the liver. Further studies and elucidation of the functions of the phosphoproteins should be both a challenging and an interesting field of study.

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PROTEIN KINASE ACTIVITY AND ENDOGENOUS PHOSPHORYLATION IN SUBFRACTIONS OF RAT LIVER MITOCHONDRIA

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Summary

Rat liver mitochondria were subfractionated into outer membrane, intermembrane and mitoplast (inner membrane and matrix) fractions. Of the recovered protein kinase activity, 80–90% was found in the intermembrane fraction, while the rest was associated with mitoplasts. The intermembrane protein kinase was stimulated by cyclic AMP, while the mitoplast enzyme was stimulated by the nucleotide only after treatment with Triton X-100. Extracted protein kinase resolved into three peaks on DEAE-cellulose chromatography. All three peaks were present both in the intermembrane fraction and in mitoplasts. One peak corresponded to the catalytic subunit of cyclic AMP-dependent protein kinases, one was a cyclic AMP-independent enzyme, and the third was the cyclic AMP-dependent type II enzyme. The endogenous incorporation of phosphate was particularly high in the outer mitochondrial membrane, and occurred also in the mitoplast fraction. The incorporation in mitoplasts was to a double band of M_r 47 500, and in outer membranes to apparently heterogeneous material of comparatively low molecular weight.

Introduction

In rat liver cells protein kinase activity and endogenous substrates are present both in the soluble fraction [1,2] and in various particulate fractions [3,4] including mitochondria [5,6]. Protein kinase activity has been found attached to the inner mitochondrial membrane of mouse heart and liver cells [7], but the complete submitochondrial distribution of the enzyme is still unclear. The protein kinase of rat liver mitochondria has been separated into two fractions

by DEAE-cellulose chromatography [8,9], of which one fraction is stimulated by cyclic AMP. There is some evidence that mitochondrial and cytosolic protein kinase have similar properties [10]. The endogenous phosphorylation of mitochondrial proteins has not been studied in detail. Apart from pyruvate dehydrogenase which is phosphorylated by a specific protein kinase [11], very little is known about how many proteins may become phosphorylated in mitochondria and what their submitochondrial localisation and identity is.

We wish to report here on the distribution of protein kinases and substrates for these enzymes in mitochondrial subfractions. Evidence for the identity of mitochondrial protein kinases with that of protein kinases found in other subcellular fractions is presented.

Materials and Methods

Chemicals. [γ - 32 P]ATP was obtained from The Radiochemical Centre (Amersham). Protamine sulfate, α -casein, calf thymus histones (Type II-A) and bovine serum albumin (A 6003) were from Sigma. Acrylamide from Eastman was recrystallised twice from acetone before use.

Preparation of mitochondria. Male Sprague-Dawley rats fed ad libitum were stunned by a blow on the head and bled to death by cutting the carotic artery. Livers were excised immediately and dropped into ice-cold isolation medium (2 mM Hepes, pH 7.5, 220 mM mannitol, 70 mM sucrose and 0.5 mg/ml of bovine serum albumin). All preparations were performed at 0–4°C.

Mitochondria were isolated by the method of Greenawalt [12]. Their purity was examined by marker enzymes analyses. In several preparations the average relative specific activities compared to the tissue homogenate of cytochrome *c* oxidase, NADPH-cytochrome *c* reductase and lactate dehydrogenase were 4.1, 0.6 and 0.06, respectively. Thus the contamination of the preparation by endoplasmic reticulum and cytosolic material was small.

Subfractionation of mitochondria. The mitochondria were subfractionated into outer membrane, intermembrane and mitoplast (inner membrane and matrix) fractions by a procedure modified from Parsons and Williams [13]. Mitochondria from 35–50 g of liver were suspended in isolation medium to a volume of 9 ml or less by drawing the material into a pipette a few times. They were subjected to swelling in a hypotonic medium (20 mM sodium phosphate buffer, pH 7.2, and 0.2 mg/ml of bovine serum albumin). After centrifugation at 35 000 $\times g$ for 20 min and resuspension of the pellet in the same manner in hypotonic medium the crude mitoplast fraction was sedimented at 1900 $\times g$ for 15 min, while the crude outer membrane fraction remained in the supernatant. Outer membranes were pelleted at 35 000 $\times g$ for 20 min. This fraction and the mitoplasts were suspended (outer membranes in a glass-Teflon homogeniser and the mitoplasts as described above) in the phosphate buffer and subjected to sucrose density gradient centrifugation for 90 min at 65 000 $\times g$. The gradients were loaded in Beckman SW 27 tubes and consisted of 7 ml of 23.2% (w/v), 10 ml of 37.7% and 7 ml of 51.3% sucrose in the phosphate buffer. The outer membrane fraction was recovered in the interface between the 23.2 and 37.7% sucrose layers. It was diluted with isolation medium and sedimented at 105 000 $\times g$ for 1 h. This fraction and the mitoplasts, which were recovered

from the sucrose gradient as a pellet, were suspended in isolation medium. The intermembrane fraction (the soluble fraction located between the outer and inner mitochondrial membranes) was obtained from the supernatant of the first centrifugation at $35\,000 \times g$ and was clarified by centrifugation at $105\,000 \times g$ for 1 h.

The purity of the mitochondrial subfractions was examined using the following marker enzymes: outer membranes, monoamine oxidase (analysed by the method of [14]); intermembrane fraction, adenylate kinase [15]; mitochondria, cytochrome *c* oxidase (measured as described in [16] but using an extinction coefficient of $21\text{ mM}^{-1} \cdot \text{cm}^{-1}$ [17]) from the inner membrane and glutamate dehydrogenase [18] from the matrix. The specific activities of the marker enzymes from a representative experiment are shown in Table II. It may be seen that there was some cross-contamination between the outer membrane fraction and the mitochondria, and that there had been a leakage of matrix material into the soluble intermembrane fraction during the preparation procedure.

As an alternative we subfractionated the mitochondria using digitonin [12]. The resolution between the subfractions was not improved by this fractionation procedure, however, and there was a substantial release of monoamine oxidase into the intermembrane fraction.

Analyses. Protein kinase analyses were performed essentially as described earlier [19]. The incubation mixture contained Tris-HCl, $5\text{ }\mu\text{mol}$, pH 7.8; MgCl_2 , $1\text{ }\mu\text{mol}$; NaCl, $30\text{ }\mu\text{mol}$ (when protamine was used as substrate); dithioerythritol, $0.1\text{ }\mu\text{mol}$; [$\gamma\text{-}^{32}\text{P}$]ATP, 50 nmol (specific activity $30\,000\text{--}100\,000\text{ dpm/nmol}$); substrate, 0.1 mg ; and enzyme protein in a total volume of $100\text{ }\mu\text{l}$. The cyclic AMP concentration was $5\text{ }\mu\text{M}$ when present. Incubations were for 10 min at 20°C , and the substrate used was protamine unless otherwise indicated. Under these conditions the reaction was linear with time and amount of enzyme material, indicating that the ATP concentration was sufficient. Incubations were stopped by the addition of $10\text{ }\mu\text{l}$ 12 M HCl and the mixture was transferred quantitatively to filter paper discs (Whatman 3MM). The discs were washed in hot trichloroacetic acid/tungstate solution followed by ethanol and ether as described earlier in detail [20]. Endogenous phosphorylation was measured as hot trichloroacetic acid/tungstate-precipitable material insoluble in ethanol and ether by a similar procedure excluding protamine and NaCl from the reaction mixture [21]. Protein was measured by the Lowry method [22] after precipitation of samples in 10% trichloroacetic acid. Bovine serum albumin was used as a standard. All determinations were performed in duplicate or triplicate.

DEAE-cellulose chromatography and isoelectric focusing. Protein kinase patterns were obtained by DEAE-cellulose chromatography (DE-52, Whatman) after solubilisation of the membrane in 2% Triton X-100 as described earlier [23]. A $1.5 \times 25\text{ cm}$ column was equilibrated with buffer (10 mM Tris-HCl, pH 7.4, 1 mM MgCl_2 , 0.1% (w/v) Triton X-100, and 0.1 mM dithioerythritol). Samples were dialysed against the same buffer prior to chromatography. After application of a sample the column was washed with buffer before elution of protein kinase with a linear salt gradient running from 0 to 0.4 M KCl in a total volume of 500 ml buffer. Individual protein kinase peaks were analysed

further by isoelectric focusing [23]. Determinations of isoelectric points were performed twice or three times.

SDS-polyacrylamide gel electrophoresis. Endogenous phosphorylation for gel electrophoresis was performed with 100 μg of membrane protein in a total volume of 50 μl . The specific activity of [γ - ^{32}P]ATP was approximately 10^6 dpm/nmol. After 10 min trichloroacetic acid was added to a final concentration of 10%. The membranes were sedimented and washed in 10% trichloroacetic acid to remove excess radioactivity. Care was taken to remove as much acid as possible before suspension of the pellets in 50 μl of water containing bromophenol blue as an indicator. When necessary the suspension was titrated to blue color with 1 M NaOH. Then an equal amount of denaturing medium was added to give the following final concentration: SDS, 2% (w/v); 2-mercaptoethanol, 5% (v/v); sucrose, 5% (w/v); Na_2EDTA , 2.5 mM; Tris, 0.041 M; and boric acid, 0.04 M; pH was adjusted to 8.64 with NaOH. Immediately prior to electrophoresis the samples were immersed in boiling water for 3 min and then cooled to room temperature.

SDS-polyacrylamide gel electrophoresis was performed in slab gels with a discontinuous buffer system [24]. The pH 9.18 buffer was used in the separation gel. The separation gel had a total monomer content of 13.3% and the proportion of *N,N'*-methylene bisacrylamide was 1.0%. Corresponding values for the large pore gel were 4.4% and 5.7%, respectively. In one case the buffer system of Laemmli [25] was used. Then the total monomer content was 10% and the proportion cross-linker 2.5%. Gels were cast in perspex moulds fitting the Pharmacia GE4 electrophoresis apparatus. Electrophoresis was carried out at a constant current of 20–25 mA/gel until the visible salt front reached the bottom (usually 3–3.5 h). Gels were stained for 15 h in 0.1% Coomassie brilliant blue dissolved in 50% (v/v) methanol and 7% (v/v) acetic acid. They were destained in a Pharmacia GD-4 destainer for 1 h and washed in several changes of 25% methanol and 7% acetic acid. Destained gels were dried in a BioRad gel drier. They were then autoradiographed using Osray M3 X-ray films (Agfa-Gevaert). The following standard proteins were used: bovine serum albumin, M_r 69 000; ovalbumin, M_r 45 000; soybean trypsin inhibitor, M_r 21 500 and sperm whale myoglobin, M_r 17 200.

Results

Distribution of protein kinase activity and endogenous phosphorylation in mitochondrial subfractions

Purified mitochondria contain protein kinase activity and will support endogenous incorporation of phosphate into trichloroacetic acid-precipitable material. The submitochondrial distribution of these activities was examined (Table I). Most of the protein kinase activity was recovered in the intermembrane fraction (approximately 80% when analysed in the absence and 90% in the presence of cyclic AMP). The remaining enzyme appeared in the mitoplasts, except for a small amount in the outer membrane fraction. The total protein kinase activity of the mitochondrial subfractions was 2–4 times higher than that of the parent mitochondria. We have observed a similar increase in enzyme activity upon treatment of intact mitochondria with detergents, indicating that

TABLE I

PROTEIN KINASE ACTIVITY AND ENDOGENOUS PHOSPHORYLATION IN SUBMITOCHONDRIAL FRACTIONS

Each experiment includes materials from three rats. Protein kinase activity was determined in the presence or absence of 5 μ M cyclic AMP using protamine as substrate. Endogenous phosphorylation was not affected by cyclic AMP, and was measured in the absence of the nucleotide.

Fraction	Protein kinase activity				Endogenous phosphorylation	
	Specific ($\text{pmol} \cdot \text{min}^{-1} \cdot$ protein)		Total ($\text{pmol} \cdot \text{min}^{-1} \cdot$ g^{-1} liver)		Specific ($\text{pmol} \cdot \text{mg}^{-1}$ protein)	Total ($\text{pmol} \cdot \text{g}^{-1}$ liver)
Cyclic AMP:	—	+	—	+		
Experiment 1						
Mitochondria	7.0	8.7	55.6	68.9	50	397
Mitoplasts	3.4	4.3	22.2	28.9	15	79
Intermembrane fraction	54	100	113	218	0	0
Outer membrane	25	27	1.6	1.7	600	31
Experiment 2						
Mitochondria	2.0	2.5	16.4	21.3	26	301
Mitoplasts	2.4	2.3	12.6	12.4	15	98
Intermembrane fraction	42	70	70.3	117	0	0
Outer membrane	22	17	0.8	0.7	240	7

the outer membrane prevents enzyme-substrate interactions.

Cyclic AMP stimulated protein kinase activity by 20–30% in different mitochondrial preparations. Most of the cyclic AMP-dependent enzyme seemed to reside in the intermembrane fraction where enzyme activity was doubled by the nucleotide. The protein kinase associated with mitoplasts was cyclic AMP independent. However, after treatment of the mitoplasts with Triton X-100 a stimulation of around 30% by cyclic AMP was observed. There was also a latency of protein kinase activity in the mitoplasts, and the activity increased between 50 and 100% on detergent treatment.

The bimodal distribution of protein kinase in mitoplasts and the intermembrane fraction appears to be genuine. This is indicated by the distribution of marker enzymes in the mitochondrial subfractions (Table II). The intermembrane fraction did not contain inner membrane material. The contamination of the intermembrane fraction by matrix material is not significant enough to indicate a matrix origin of the protein kinase in the intermembrane fraction.

The distribution of endogenous phosphorylation was different from that of protein kinase activity. Endogenous phosphorylation into trichloroacetic acid/tungstate-precipitable and ethanol and ether-insoluble material occurred in mitoplasts and in the outer membrane fraction where the specific incorporation of phosphate was particularly high (Table I). The isolated intermembrane fraction, which contained most of the protein kinase activity, showed no endogenous phosphate incorporation even after a substantial concentration of this rather dilute fraction. The recovery of endogenous phosphorylation in the isolated subfractions was only 30%, compared to well above 100% for protein kinase activity. This may indicate that enzyme and substrate had been

TABLE II

DISTRIBUTION OF MARKER ENZYMES IN SUBMITOCHONDRIAL FRACTIONS

Livers from three rats were combined and subfractionated as described under Materials and Methods. Enzyme analyses were performed in duplicate. All enzyme activities are expressed in $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ protein.

Fraction	Protein (mg/g liver)	Monoamine oxidase	Cytochrome c oxidase	Adenylate kinase	Glutamate dehydrogenase
Mitochondria	8.1	5.8	300	270	680
Mitoplasts	5.2	3.6	520	0	690
Intermembrane fraction	1.85	0.35	0	1000	160
Outer membrane	0.051	95	55	0	51

separated in part through the fractionation procedure.

The distribution of endogenous phosphorylation in the isolated submitochondrial fractions was very similar to that of monoamine oxidase, the marker enzyme for outer mitochondrial membranes. An analysis of the products of endogenous phosphorylation by SDS-polyacrylamide gel electrophoresis and autoradiography (Fig. 1) showed a double band of M_r 47 500 in the mitoplast fraction, while the phosphorylated material in the outer membrane was of rather low molecular weight. Since the M_r 47 500 bands and the low molecular

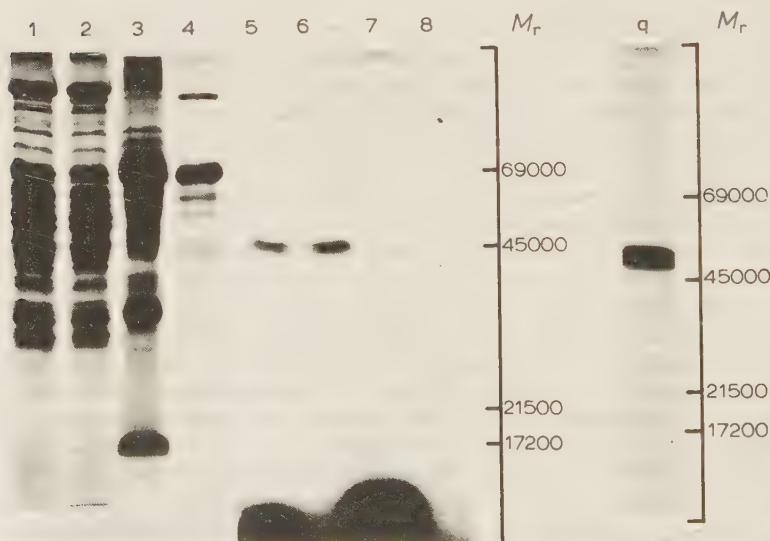


Fig. 1. Protein and phosphorylation patterns in submitochondrial fractions. Isolated fractions were incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and subjected to SDS-polyacrylamide gel electrophoresis. 1-4, protein patterns of mitochondria, mitoplasts, outer membranes and intermembrane fractions, respectively. 5-8, phosphoprotein patterns of corresponding fractions. 9, phosphoprotein pattern of mitoplasts obtained after electrophoresis by the method of Laemmli [25]. The strong protein band at M_r 69 000 in the intermembrane fraction and outer membranes is bovine serum albumin from the isolation medium.

weight material occurred together after phosphorylation of unfractionated mitochondria, it appears that they were separated efficiently by the fractionation procedure. Therefore, the location of the M_r 47 500 components is likely to be in the mitoplast fraction, and the location of the low molecular weight material preferentially in outer membranes.

tain, but it appears to be heterogeneous due to its broad appearance in the gel. It is unlikely that this material consists of contaminating [γ - ^{32}P]ATP or [^{32}P]-phosphate because of its retardation on electrophoresis in dense polyacrylamide gels, its chromatographic properties in Sephadex G-100 in the presence of 1% SDS (K_{av} = 0.15), or its behavior on high-voltage electrophoresis where it is well separated from ATP and inorganic phosphate. It is also less likely that the phosphorylated material consists of phospholipids, which have other separation properties on thin-layer chromatography and, furthermore, are removed in the washing procedure used to analyse endogenous phosphorylation. The material may consist of phosphorylated polypeptides, although we have no firm evidence for this so far.

Identification of protein kinases in mitochondria and mitochondrial subfractions

Isolated mitochondria were treated with Triton X-100, and the solubilised material was chromatographed on DEAE-cellulose. Protein kinase activity resolved into three peaks (Fig. 2A). None of the peaks was stimulated significantly by cyclic AMP when eluted from the column. The elution properties of the peaks were the same as those of the three protein kinase fractions identified in microsomes [23] and three of the four enzyme fractions present in the cytosol [23]. Thus, the first peak (MI) should be the catalytic subunit of cyclic AMP-dependent protein kinases. MII should be a cyclic AMP-independent enzyme, and MIII the type II cyclic AMP-dependent protein kinase (see below).

To further test the identity of the peaks they were subjected to isoelectric

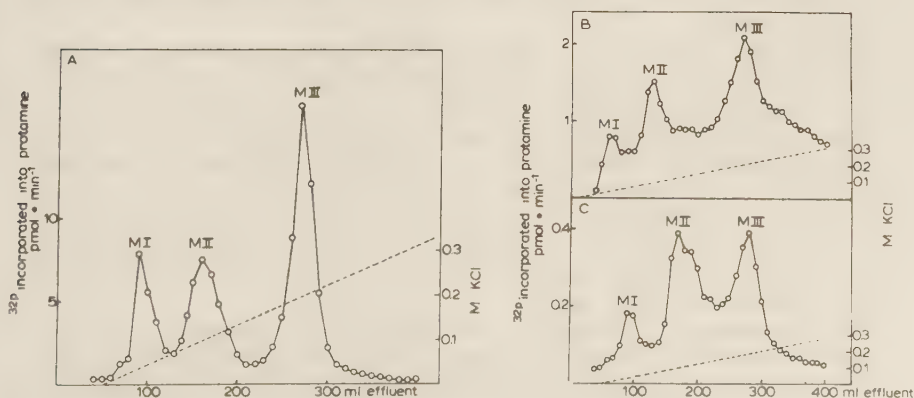


Fig. 2. DEAE-cellulose chromatography of mitochondrial protein kinase activity. Protein kinase activity was determined with protamine as substrate. No significant effect of cyclic AMP on protein kinase activity in any of the peaks was observed. (A) Mitochondria; (B) intermembrane fraction, and (C) mitoplasts.

TABLE III

ISOELECTRIC POINTS OF PROTEIN KINASE FRACTIONS

Isoelectric focusing was performed as described earlier [23]. Samples were run with or without cyclic AMP treatment or after pretreatment with 0.1 mM cyclic AMP and 10 mM theophylline for 15 min at 20°C. The designations of the cytosolic protein kinases are the same as in [23].

Protein kinase fraction	Isoelectric point	
	No cyclic AMP	+ cyclic AMP
MI	8.05	8.15
MII	5.45	5.45
MIII	4.75	8.20
		7.15
		4.70
CI	8.10	8.15
CIIf	5.45	5.45
CIII	4.90	8.15
		7.35

focusing (Table III). The isoelectric points of each of the mitochondrial enzyme fractions are similar to the isoelectric points of corresponding enzyme fractions from microsomes or the cytosol (cf. Ref. 23) when focused both in the presence and absence of cyclic AMP. Although cyclic AMP did not stimulate the activity of fraction MIII appreciably before focusing, the fraction behaved as the undissociated enzyme and no free catalytic subunit could be detected when focused in the absence of the nucleotide. The lack of stimulation by cyclic AMP was probably due to dissociation of the enzyme in the assay medium. It has been noted earlier that substrates for cyclic AMP-dependent protein kinases tend to dissociate these enzymes [26,27]. It is likely that such a dissociation would be facilitated at the low enzyme concentrations used in the isoelectric focusing experiments and in analyses of eluates from DEAE-cellulose columns.

The substrate specificities of the protein kinase fractions were also tested (Table IV). Protamine was the best substrate for all three enzyme fractions, while calf thymus histones and especially α -casein were poor as substrates. After concentration of the DEAE eluate the MIII enzyme fraction was activated by cyclic AMP with protamine or histones as substrate. It is also seen that the MI and MIII fractions require NaCl for maximal enzyme activity, while the MII fraction is unaffected by the salt. The substrate specificity of each of the mitochondrial enzymes thus corresponds closely to the specificities of the enzymes from microsomes and the cytosol that elute from DEAE-cellulose under the same conditions.

The chromatography profiles of protein kinase from the intermembrane and mitoplast fractions were similar to each other and to the profile of unfractionated mitochondria with three peaks eluting at similar KCl concentrations (Fig. 2). Mitoplasts, however, contained relatively more of the MII enzyme fraction.

Discussion

We have shown here that approximately 85% of the total protein kinase activity associated with mitochondria is present in the intermembrane fraction while the rest is associated with the mitoplasts. This distribution of the enzyme appears to reflect the situation in isolated mitochondria since isolated mitoplasts did not contain any adenylate kinase activity (marker enzyme for the intermembrane fraction), and since very little of the mitoplast markers were found in the intermembrane fraction. The possibility that the mitoplast protein kinase became associated with the intermembrane fraction during the isolation procedure seems less likely since the enzyme activity was partly masked in isolated mitoplasts. Furthermore, the masked enzyme which became unmasked on addition of Triton X-100 was not sensitive to trypsin treatment (results not shown). The increase in enzyme activity on addition of Triton X-100 was not due to the stimulatory effect of detergents on protein kinase activity observed by Sugden et al. [28]. This effect is seen when histones but not when protamines are used as substrates.

An important question is whether the protein kinase found in mitochondria, and particularly in the intermembrane fraction, is a cytosolic contaminant. The relative specific activity of the cytosolic marker enzyme lactate dehydrogenase in isolated mitochondria was 0.06 relative to the tissue homogenate and most of the activity was found in the intermembrane fraction after subfractionation of the mitochondria. Only around 2–3% of the total protein kinase activity of rat liver homogenates was associated with highly purified mitochondria, and the relative specific activity was approximately 1. The higher relative specific activity in mitochondria for protein kinase than for lactate dehydrogenase indicates a mitochondrial localisation (also in the intermembrane fraction) of protein kinase. Other observations tend to support this idea. Thus, no cyclic AMP-dependent type I protein kinase [29], which is abundant in rat liver cytosol [23], was found in mitochondria. This enzyme was expected to be present in the intermembrane fraction if an unspecific association of cytosolic protein kinase to mitochondria occurred during the preparation procedure.

The presence of protein kinase activity in the intermembrane fraction is puzzling since no phosphorylatable proteins were found there. Apart from the possibility discussed above concerning an artefactual association of cytosolic protein kinase with this fraction, there may be other explanations. One is that the intermembrane enzyme participates in the phosphorylation of proteins exposed on the inner mitochondrial membrane. Another is that the pattern of protein phosphorylation *in vitro* differs from that *in vivo*. This has been shown to be the case in the cytosol where three times more proteins become phosphorylated upon incubation of hepatocytes with [^{32}P]phosphate than when isolated cytosol is incubated with [$\gamma\text{-}^{32}\text{P}$]ATP [30]. This would also explain why there are several kinase species in the mitochondrial subfractions but only one or two substrates can be recognised after phosphorylation *in vitro*.

In an earlier investigation Kleitke et al. [8] found that highly purified mitochondria contained a partly latent protein kinase activity and that this became cyclic AMP dependent after solubilisation with Triton X-100. This is in accor-

dance with the results presented here with purified mitoplasts. In another investigation Vardanis [7] found that mouse liver and heart mitochondrial protein kinase was associated with the inner membrane. He did not detect any activity in the intermembrane fraction. No control experiments were presented, however, to ascertain whether there had been any leakage of intermembrane material during the mitochondrial preparation procedure. It was also found [7,9] that the protein kinase activity of the inner membrane increased 10–20-fold when it was released by sonication or freezing and thawing, and that the activity was cyclic AMP independent. We have not been able to release kinase activity by freezing and thawing or sonication by the methods described.

Histones were reported to be much superior to protamine as substrate for the mitochondrial protein kinase [7], while we obtained the opposite result. We have found that this discrepancy is due to an incomplete recovery of the phosphorylated protamine when trichloroacetic acid alone, rather than trichloroacetic acid/tungstate as here, is used as precipitant in the kinase analysis. A comparison shows that the specific activity of the mitochondrial protein kinase was rather similar in all the investigations when histones were used as substrates (when the different incubation temperatures used were taken into account).

Earlier investigations [8,9] report on two protein kinase fractions in rat liver mitochondria. Most likely they correspond to our fractions MI and MIII. Since histones are poor substrates for the MII fraction, this enzyme was not detected in the earlier studies.

It is interesting that the same three protein kinases found in mitochondria are found also in other subcellular fractions such as cytosol, microsomes and plasma membranes [4,23]. There is a difference in relative amounts of the protein kinases when mitochondria are compared with the other fractions. Mitochondria contain relatively more of the free catalytic subunit (MI) and relatively little of the cyclic AMP-independent enzyme (MII). It is also interesting to note that the cyclic AMP-dependent type I protein kinase is present only in the cytosol and not in any of the particulate fractions examined so far in rat liver cells [4,23]. The type II enzyme on the other hand, is found both in the cytosol and in particulate fractions including mitochondria, microsomes and plasma membranes [4]. Perhaps this difference in subcellular distribution between the type I and type II protein kinases reflects different functions for them. It is not clear why these two enzymes with the same substrate specificity exist together in the cell. We have found earlier in reconstitution experiments that the isolated type II enzyme, but not the type I enzyme or the free catalytic subunit, will bind to smooth microsomes [23]. Since the difference between these enzyme fractions resides in the regulatory subunit, it is possible that the regulatory subunit of the type II enzyme will determine the distribution of the protein kinase in the cell.

The question about the identity of the phosphorylated components in the mitoplasts is still open. It is known that pyruvate dehydrogenase undergoes regulatory phosphorylation-dephosphorylation cycles [11], but we have not been able to confirm whether one of the two phosphoprotein fractions observed corresponds to pyruvate dehydrogenase. There are no reports on the isolation of this enzyme in a pure form from rat liver, but the bovine heart

enzyme has an M_r of 41 000 [31] which is rather lower than our phosphoproteins. It has also been reported that NAD-dependent isocitrate dehydrogenase activity is regulated by cyclic AMP, perhaps through a phosphorylation reaction [32].

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Localization of Protein Kinase Activity and Phosphoproteins in Mitoplasts from Rat Liver

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Rat liver mitoplasts (inner mitochondrial membrane and matrix) contain protein kinase activity. This activity increases twofold on addition of Triton X-100. The activity observed in absence of Triton X-100 is probably exposed on the outer surface of mitoplasts, since it is sensitive to trypsin treatment. Most of the remaining protein kinase is bound to the membrane fraction, presumably on the inside of (or else hidden in) the inner mitochondrial membrane. Only a small part of the kinase activity is found in the mitochondrial matrix. A phosphoprotein band, partly resolved into a doublet, was observed on electrophoresis in SDS-polyacrylamide gels after endogenous phosphorylation of mitoplasts, inner mitochondrial membrane or matrix. When isolated fractions are phosphorylated approximately 75% of the phosphoprotein is found in the matrix, and the remainder in the inner membrane. The phosphorylation of the doublet is inhibited by inhibitors to pyruvate dehydrogenase kinase, suggesting that it represents the phosphorylated subunit of pyruvate dehydrogenase.

In rat liver cells protein kinases and endogenous substrates for these enzymes have been found both in soluble^{1,2} and particulate^{3,4} fractions including mitochondria.⁵⁻⁸ The mitochondrial protein kinase activity is distributed in various submitochondrial fractions, including outer membranes, the intermembrane fraction and mitoplasts.⁸ This enzyme can be separated into 2 or 3 subfractions by DEAE-cellulose chromatography.^{8,9}

Although various mitochondrial functions are under hormonal regulation, which often involves phosphorylation/dephosphorylation of specific proteins, comparatively little is known about protein phosphorylation in liver mitochondria. The only mitochondrial phosphoproteins detected in

our earlier study⁸ were located in the mitoplast fraction. In other tissues pyruvate dehydrogenase is the only mitochondrial protein which is known to be phosphorylated.¹⁰⁻¹² This report concerns the further localization of protein kinases and phosphoproteins within the ~~inner~~ mitoplast fraction. *Him* Evidence is presented that the mitoplast phosphoproteins represent pyruvate dehydrogenase.

EXPERIMENTAL

Chemicals. Inorganic [³²P]-phosphate was obtained from the Radiochemical Centre, Amersham. [γ -³²P] ATP was synthesized according to Chang *et al.*¹³ Protamine sulfate, trypsin, bovine serum albumin (A 6003) and altractyloside were from Sigma. Acrylamide was from Bio-Rad and *N,N'*-methylene bisacrylamide (twice recrystallized from acetone) from Eastman. All other chemicals were of reagent grade.

Methods. Mitoplasts were prepared as described earlier.⁸ They were subfractionated into inner membranes and matrix as follows. Mitoplasts were suspended in distilled water and frozen and thawed 3 times, followed by centrifugation at 35 000 $\times g$ for 20 min. After decanting the supernatant, the above procedure was repeated once. The combined supernatants contained the mitochondrial matrix material, and the pellet consisted of the inner membrane as judged by the distribution of the marker enzymes cytochrome oxidase (inner membrane) (measured as described in Ref. 14, but using an extinction coefficient of 21 mM⁻¹ cm⁻¹)¹⁵ and glutamate dehydrogenase¹⁶ (matrix). The result of the subfractionation and the degree of crosscontamination is shown in Table 1.

Protein kinase activity was determined as described earlier.^{8,17,18} Protein was measured by

Table 1. Distribution of marker enzymes in subfractionated mitoplasts. Liver from three rats were combined and mitoplasts were prepared and subfractionated as described in Experimental. Enzyme analyses were performed in duplicate. Enzyme activities are expressed as $\mu\text{mol min}^{-1} \text{mg}^{-1}$ protein.

Fraction	Protein (mg/g liver)	Cytochrome c oxidase	Glutamate dehydro- genase
Mitoplasts	1.96	2.29	0.921
Inner membrane	1.01	2.08	0.163
Matrix	0.88	0.022	2.17

the Lowry method¹⁹ after precipitation with 7% trichloroacetic acid in the presence of 0.015% sodium deoxycholate.²⁰ Bovine serum albumin was used as a standard.

Proteolytic digestion of mitoplasts (protein concentration 7–12 mg/ml) was performed with trypsin using a trypsin to protein ratio of 1:50 (w/w).²¹ The digestion proceeded for 18 h at 0°C and was stopped by addition of a threefold excess (w/w) of soybean trypsin inhibitor.

Endogenous phosphorylation for electrophoresis was performed as described earlier⁸ using 50 μg of protein in a total volume of 50 μl . The incubation time was 5 min.

Sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis was performed in slab gels using the buffer system of Laemmli.²² The total monomer content of the separation gel was 10% and the proportion of crosslinker was 2.5%. The electrode buffer contained 0.0083 M Tris and 0.192 M glycine (pH 8.1). The procedure for electrophoresis and autoradiography has been described in detail earlier.⁸ The following standard proteins were used: bovine serum albumin, M_r 69 000; ovalbumin, M_r 45 000; soybean trypsin inhibitor, M_r 21 500; and sperm whale myoglobin, M_r 17 200.

Endogenous phosphorylation of proteins was measured by cutting out the phosphoprotein bands from SDS-polyacrylamide gels and counting the pieces in a liquid scintillation counter using 0.4% Omnifluor (New England Nuclear) in toluene as scintillation liquid. The doublet band at M_r 44 000–45 000 was counted as one band. The blank used was an equal size part of the gel from a corresponding region without phosphoprotein bands.

When testing the influence of effectors of pyruvate dehydrogenase kinase on the phosphorylation of proteins the conditions of Morgan and Routtenberg¹² were used. After incubation the

samples were subjected to electrophoresis and scintillation counting as described above. Tests were run in triplicate.

The influx of ATP into mitoplasts was measured by incubation in the following mixture: 50 mM Tris-HCl, pH 7.7, 10 mM MgCl_2 , 1 mM dithioerythritol, 0.5 mM [γ -³²P] ATP (10 000–30 000 dpm/nmol) and 5 mg/ml mitoplastic protein. To study the time-dependency 50 μl samples were withdrawn at appropriate times and the reaction was stopped by mixing with an equal volume of 5 mM atractyloside (a potent ATP carrier inhibitor), 2 mM HEPES, pH 7.5, 0.22 M mannitol and 0.07 M sucrose. This mixture was layered over 200 μl of 5 mM atractyloside, 2 mM HEPES, pH 7.5, 0.22 M mannitol and 0.65 M sucrose in a 400 μl centrifuge tube to a Beckman Microfuge. The sample was centrifuged for 1 1/2 min and the supernatant was carefully removed. The pellet was then counted in 5 ml Biofluor (New England Nuclear). The influx of ATP was linear for at least 10 min.

RESULTS

Localization of protein kinase. As we have previously shown⁸ purified rat liver mitoplasts possess a protein kinase activity that can phosphorylate exogenous substrates. The phospho-

Table 2. Effect of trypsin treatment on protein kinase activity in mitoplasts. Intact mitoplasts, or mitoplasts solubilized in 1% Triton X-100, were incubated with trypsin for 18 h at 0°C as described in Experimental. Protein kinase assays were performed in the absence or presence of 1% Triton X-100. Each experiment includes the combined material from three rats.

	Protein kinase activity (pmol min ⁻¹ mg protein ⁻¹)	
	-	+ 1% Triton X-100
Experiment 1		
Mitoplasts	10.2	22.3
Mitoplasts + trypsin	2.2	13.5
Solubilized mitoplasts + trypsin		4.6
Experiment 2		
Mitoplasts	11.8	23.4
Mitoplasts + trypsin	3.5	16.5
Solubilized mitoplasts + trypsin		6.2

Table 3. Protein kinase activity and endogenous incorporation of [^{32}P]-phosphate into proteins of subfractionated mitoplasts. Each experiment includes material from three rats. Protein kinase activity was determined in the presence or absence of $5\ \mu\text{M}$ cyclic AMP using protamine as a substrate. The endogenous incorporation of phosphate was not affected by cyclic AMP and was measured in the absence of the nucleotide.

Fraction	Protein kinase activity				Endogenous incorporation of [^{32}P]-phosphate	
	Specific (pmol min mg^{-1} protein)		Total (pmol min g^{-1} liver)		Specific (pmol mg^{-1} protein)	Total (pmol g^{-1} liver)
	-	+	-	+ cAMP		
Experiment 1						
Mitoplasts	6.14	6.98	15.7	17.7	30.6	108
Mitoplasts + 1% Triton X-100	9.50	13.2	24.3	33.8	—	—
Inner membrane	9.52	10.5	11.3	12.5	8.7	13.4
Inner membrane + 1% Triton X-100	19.6	22.0	23.3	26.1	—	—
Matrix	1.89	1.66	2.2	2.0	28.5	46.6
Experiment 2						
Mitoplasts	10.4	11.9	20.4	23.3	33.8	183
Mitoplasts + 1% Triton X-100	19.2	23.4	37.6	45.9	—	—
Inner membrane	16.3	18.5	16.5	18.8	8.1	15.9
Inner membrane + Triton X-100	33.8	33.8	34.2	34.2	—	—
Matrix	4.1	6.0	3.6	5.3	17.6	33.6

rylating activity increases approximately twice on addition of Triton X-100. Presumably this is due to the fact that a part of the protein kinase is located either within the mitoplast membrane or in the mitochondrial matrix, and that the enzyme therefore cannot interact with the added protein substrate.

In order to examine this hypothesis, mitoplasts were treated with trypsin and then analyzed for protein kinase activity in the presence and absence of Triton X-100 (Table 2). The activity obtained without detergent decreased by approximately 80% upon trypsin treatment, while the latent activity (*i.e.* the increase in activity seen after the addition of Triton) was unaffected. The control experiment where mitoplasts were trypsinized in the presence of Triton X-100 showed that the latent protein kinase was equally sensitive to the protease as the exposed activity. In another control experiment where both soybean trypsin inhibitor and Triton X-100 were

present during the trypsin treatment there was no decrease in protein kinase activity (not shown). These results suggest a surface location of approximately half the protein kinase activity of mitoplasts (using protamine as a substrate), while the remaining activity is located either in the matrix or on the inside of (or else hidden in) the inner mitochondrial membrane.

The location of the protein kinase was further examined in mitoplasts subfractionated into inner membranes and matrix (Table 3). Approximately 10% of the recovered protein kinase was found in the matrix. The remaining activity appeared in the inner membrane subfraction, and showed the same latency as the protein kinase of mitoplasts. The kinase activity of the matrix was independent of detergent (not shown). The effect of cyclic AMP on enzyme activity was slight, both in mitoplasts and in the isolated subfractions.

Localization of phosphoproteins. When mitoplasts

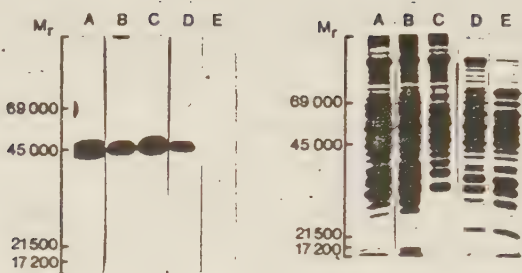


Fig. 1. Phosphoprotein and protein patterns of mitoplasts and mitoplast subfractions. Mitoplasts and subfractions were incubated with [γ - 32 P] ATP. They were subjected to SDS-polyacrylamide gel electrophoresis, and the gels were stained for protein (right) and autoradiographed (left). A, intact mitoplasts; B, inner membrane; C, matrix; D, trypsin-treated mitoplasts; E, mitoplasts solubilized in Triton X-100 and then trypsin-treated. The M_r values of standard proteins are indicated on the left.

were incubated with [γ - 32 P] ATP, radiolabelled phosphate was incorporated into two polypeptides that could be partly resolved by electrophoresis in SDS-polyacrylamide gels (Fig. 1). If mitoplasts were subfractionated and then phosphorylated, most of the recovered phosphoprotein was found in the matrix fraction (Table 3), although a substantial amount (approximately 25%) was also found with the inner membrane.

When intact mitoplasts were trypsinized before phosphorylation there was a substantial decrease in protein phosphorylation, as can be seen from the intensity of the phosphorylation bands in Fig. 1. Since most phosphoprotein was recovered in the matrix and trypsin presumably acts on the mitoplast surface this appears contradictory. However, it is possible that the ATP-translocating system might be affected by the proteolytic treatment limiting the ATP available for phosphorylation. The uptake of ATP was therefore measured in untreated and trypsin-treated mitoplasts. The uptake decreased from 104 pmol of ATP per min per mg mitoplast protein to between 30–50% of this value by the trypsin treatment. ATP may therefore be limiting in the phosphorylation of mitoplast proteins after trypsin treatment.

Identification of the phosphoprotein. The only phosphoprotein identified in mitochondria up to now is pyruvate dehydrogenase with M_r 41 000^{2,3} for the phosphorylated subunit. Morgan and Routtenberg¹² recently identified a double phosphoprotein band in rat brain mitochondria

with a similar M_r as this enzyme. Although our phosphoprotein doublet has a higher M_r (44 000–45 000) the difference is within the limits expected for determinations by SDS-polyacrylamide gel electrophoresis.

To further identify the phosphoproteins the effect on phosphate incorporation into matrix phosphoproteins of various pyruvate dehydrogenase kinase modulators was tested (Table 4). It is seen that particularly dichloroacetate and pyruvate, but also NAD, inhibit this incorporation. These results are

Table 4. Modulation of the endogenous incorporation of [32 P]-phosphate into matrix protein. The mitochondrial matrix from three rats was incubated with [γ - 32 P] ATP in the presence of the indicated compounds. The conditions of Morgan and Routtenberg¹² were used. The incorporation of [32 P]-phosphate was measured as is described in Experimental. The incorporation is expressed as percent of control incubations in the absence of effectors.

Effector	Incorporation of [32 P]-phosphate(%)	
	Exp 1	Exp. 2
None	100	100
1 mM NADH	92	81
1 mM NAD	67	59
5 mM dichloroacetic acid	8	6
5 mM sodium pyruvate	18	13

well in accordance with the results of Morgan and Routenberg,¹² and indicate that the phosphoprotein doublet in the matrix of rat liver mitochondria represents phosphorylated pyruvate dehydrogenase.

DISCUSSION

When studying the location of components in mitoplasts there are four different compartments to consider: the matrix, the outer and inner membrane surface and the lipid phase of the membrane. With the techniques used here it is possible to differentiate between three compartments (the lipid phase and the inner surface act as one compartment in these experiments).

It can be calculated from our results that approximately 90% of the total protein kinase activity of mitoplasts (using protamine as a substrate) is bound to the membrane, while the remaining activity resides in the mitochondrial matrix. Due to the increase in kinase activity on treatment of isolated inner membranes with Triton X-100 and the partial sensitivity to trypsin treatment, around 50% of the membrane-bound activity appears to be located on the outer surface, while the rest is on the inner surface or else hidden in the membrane. Kleitke *et al.*⁹ have also found a latent protein kinase in a "highly purified mitochondria" fraction which seems to be similar to our mitoplast fraction. The relative amount of latent protein kinase reported by them is similar to what we have found here. They did not investigate the location of the kinase further, however.

The phosphoprotein was recovered primarily in the matrix, but approximately 25% was found in the inner membrane fraction. This is a much higher proportion than was found in the inner membrane of the matrix marker enzyme glutamate dehydrogenase (*cf.* Table 1). It appears, therefore, that the phosphoprotein has a rather high affinity for the membrane, and may be attached functionally to the inside of the inner membrane. The endogenous phosphorylation of the mitoplast proteins decreased after trypsin treatment. This decrease was probably due to an impairment of the transport of ATP over the membrane after trypsin treatment rather than to proteolysis of phosphorylatable proteins located on the mitoplast surface.

The only mitochondrial phosphoprotein iden-

tified so far is pyruvate dehydrogenase.¹⁰ In rat brain mitochondria this enzyme appears as a phosphoprotein doublet of M_r 41 000–42 000.¹² This is lower than the M_r 44 000–45 000 we observe for the phosphoprotein doublet of rat liver mitochondria. Since there is a very similar effect of various pyruvate dehydrogenase kinase modulators on the phosphorylation of these bands in brain and liver, it is likely that the liver phosphoproteins also represent pyruvate dehydrogenase. The observed discrepancy in molecular weight is probably due to the different buffer systems used for SDS-polyacrylamide gel electrophoresis in the two cases and the choice of molecular weight marker proteins.

The reason why the phosphoprotein appears as a doublet is not understood. The phosphorylation of both bands is affected similarly by trypsin treatment of mitoplasts or by pyruvate dehydrogenase modulators, and their relative proportion is the same after phosphorylation of either mitoplasts or isolated mitoplast subfractions. The fact that pyruvate dehydrogenase can incorporate up to three phosphate groups per enzyme subunit²⁴ seems not to be the explanation for the appearance of two bands since their separation is based on differences in molecular weight rather than on charge.

In a previous paper⁸ we reported that protein kinases and phosphoproteins are distributed differently in rat liver mitochondrial fractions. The present work shows that protein kinases and phosphoproteins are also distributed differently in mitoplasts, with 90% of the kinases in the membrane fraction and 70% of the protein phosphorylation in the matrix fraction. It should be noted, however, that pyruvate dehydrogenase kinase is likely not to phosphorylate protamine or other common protein kinase substrates appreciably,^{24,25} and that the analyzed protein kinase activity therefore should be expected to phosphorylate some other substrate. Other phosphoproteins have not been observed, however, in our experiments. Whether this is due to the presence of powerful phosphoprotein phosphatases in mitochondria, or whether factors essential for phosphorylation have been removed or destroyed during the preparation procedure is not known presently. The appearance of protein kinase activity inside mitoplasts suggests that the enzyme is genuinely mitoplasmic and not an enzyme which associates with the mitoplasts during the preparation procedure.

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Protein Kinase Activity and Endogenous Protein Phosphorylation in Rat Liver Plasma Membranes

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When rat liver plasma membranes were incubated with [γ - 32 P]ATP radio-labelled phosphate was incorporated into endogenous protein and exogenous substrate by a membrane-bound protein kinase activity. A high ATP/membrane protein ratio was required for optimum incorporation conditions. Cyclic AMP did not affect the incorporation of phosphate. The protein kinase activity was extracted from the membranes by 1% Triton X-100 and a high concentration of KCl. The solubilised enzyme resolved into two fractions on DEAE-cellulose chromatography. One enzyme fraction had the same properties as the catalytic subunit of cytosolic cyclic AMP-dependent protein kinases. Endogenously phosphorylated proteins were resolved by SDS-polyacrylamide gel electrophoresis into five major and additional minor phosphorylated components. Three of the major phosphorylated components were tightly bound to the membrane material and were not extracted by 1% Triton X-100 and 1M KCl.

The activity of several proteins is regulated by enzymically catalysed phosphorylation-dephosphorylation reactions.¹ Protein phosphorylation in rat liver is a feature of most subcellular fractions,^{2–4} and protein kinases have been isolated and characterised from the cytosol,^{3,5,6} microsomes,³ mitochondria^{7–9} and nuclei.^{10,11} Comparatively little is known about protein kinases and the phosphorylation of proteins in rat liver plasma membranes. Early studies by Blat and Harel¹² showed that [32 P]phosphate was incorporated into proteins of such membranes *in vivo*, and that isolated plasma membranes catalysed the incorporation of phosphate from [γ - 32 P]ATP into endogenous proteins.¹³ Apparently, partially purified protein kinase from rat liver cytosol also catalyses the phosphorylation of plasma membrane proteins.¹⁴

We have previously examined protein phosphorylation, protein kinases and phosphoproteins in rat liver membrane fractions including microsomes and mitochondria.^{3,4,9} Our investigations have now been extended to plasma membranes.

EXPERIMENTAL

Chemicals. [32 P]-Inorganic phosphate was obtained from the Radiochemical Centre (Amersham). [γ - 32 P]ATP was synthesised according to Chang *et al.*¹³ Protamine sulfate, calf thymus histones (Type II-A) and rabbit muscle protein kinase inhibitor were from Sigma, adenosine deaminase from Boehringer, acrylamide from Bio-Rad, and N,N' -methylene bisacrylamide (which was recrystallised twice from acetone) from Eastman. All other chemicals were of reagent grade.

Preparation of plasma membranes. Male Sprague-Dawley rats fed *ad libitum* were stunned by a blow on the head and bled by cutting the carotic artery. Livers were excised immediately and chilled at 0 °C in 5 mM Tris-HCl, pH 8.0, 0.25 M sucrose and 0.1 mM dithioerythritol. All subsequent preparations were at 0–4 °C.

Plasma membranes were prepared according to Aronson and Touster.¹⁶ The N_2 fraction, which was less contaminated by other membranes than the P_2 fraction as based on marker enzyme analyses, was used. The average relative specific activities of marker enzymes in several preparations of the N_2 fraction compared to the tissue homogenate were 34 for 5'-nucleotidase (measured by method 3 in Ref. 17); 0.3 for lactate dehydrogenase;¹⁸ 0.03 for cytochrome oxidase (measured as described in Ref. 19 but using an extinction coefficient of 21 mM⁻¹ cm⁻¹);²⁰ 1.0 for NADPH-cytochrome c reductase;²⁰ and 6.0 for *N*-acetylglucosamine galactosyltransferase.²¹

Analyses. Protein kinase activity and endogenous phosphorylation were assayed as described earlier^{22,23} with the following modifications. The protein kinase assay mixture contained 2 mM [γ -³²P]ATP (50 000–100 000 dpm/nmol) and 3 μ g of membrane protein in a final volume of 50 μ l unless otherwise stated, and the incubation time was 1 min at 25 °C. Endogenous phosphorylation was determined in a medium containing 2 mM [γ -³²P]ATP (50 000–100 000 dpm/nmol), 20 mM sodium azide and 20 μ g of protein in a final volume of 50 μ l. The incubation time was 5 min at 25 °C. In the time course experiments the reaction mixture was scaled-up and samples (50 μ l) were withdrawn at indicated times.

Substrate specificity was examined using protamine or a histone mixture as substrate.²³ Protamine phosphorylation was examined in the presence and absence of 0.3 M NaCl, and the phosphorylation of histones in the absence of salt. The amount of histone was 0.6 mg per 100 μ l of incubation mixture.

The inhibition of protein kinase activity by protein kinase inhibitor²⁴ was studied using a fixed amount of enzyme and increasing the concentrations of the inhibitor. The activity was assayed in the presence of 10 μ M cyclic AMP using protamine as a substrate.

Isoelectric focusing was as described previously.³ Protein was measured by the Lowry method²⁵ after precipitation with 10 % trichloroacetic acid. Bovine serum albumin was used as a standard. All determinations were in duplicate.

Extraction of protein kinase from plasma membranes. For extraction experiments plasma membranes were incubated with 0.3 or 1 M KCl in the presence or absence of 1 % Triton X-100. After incubation for 30 min at 0 °C samples were centrifuged at 105 000 $\times g$ for 90 min. The supernatants and pellets resuspended in buffer (10 mM Tris-HCl, pH 8.0, 1 mM MgCl₂, 0.1 mM dithioerythritol) were analysed for protein kinase, endogenous incorporation of phosphate, 5'-nucleotidase and protein content.

DEAE-cellulose chromatography. Plasma membranes were extracted in 1 % Triton X-100 and 1.0 M KCl as described above. The extract was dialysed extensively against 10 mM Tris-HCl, pH 7.4, 1 mM MgCl₂ and 0.1 mM dithioerythritol. The dialysed extract was applied onto a DEAE-cellulose (Whatman DE-52) column and the chromatography was developed with a linear KCl gradient.³ The eluate was analysed for protein kinase activity using 0.5 mM [γ -³²P]ATP and protamine sulfate as a substrate.²⁶

SDS-polyacrylamide gel electrophoresis. Endogenous phosphorylation for electrophoresis was with 20–100 μ g of membrane protein as described

above using [γ -³²P]ATP with a specific activity of 0.8×10^6 dpm/nmol. Electrophoresis was according to Neville²⁶ with some modifications.⁹ The further treatment of the gels and autoradiography have been described previously.⁹ The following standard proteins were used: bovine serum albumin, M_r 69 000; ovalbumin, M_r 45 000; soybean trypsin inhibitor, M_r 21 500; and sperm whale myoglobin, M_r 17 200.

RESULTS

Time course of protein kinase activity and endogenous phosphorylation. In initial experiments we found that purified plasma membranes from rat liver catalysed the incorporation of phosphate from ATP into protamine and that there was also incorporation into endogenous substrates. These experiments further indicated that a much higher ATP/membrane protein ratio was required in plasma membranes than in other membranes from rat liver cells to obtain optimum conditions for the incorporation of phosphate into both exogenous and endogenous substrates.

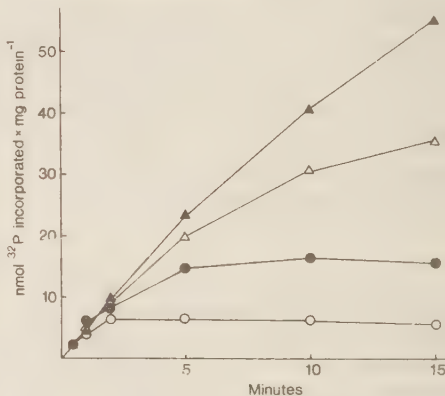


Fig. 1. Time course of plasma membrane protein kinase activity. Plasma membranes (3 or 20 μ g of membrane protein per 50 μ l incubation mixture) were incubated with protamine as exogenous substrate as described under Experimental, and the incorporation into trichloroacetic acid-sodium tungstate precipitable phosphate was determined. Analyses were performed in presence (closed symbols) or absence (open symbols) of 20 mM sodium azide. Δ , 3 μ g of membrane protein; $\circ$, 20 μ g of membrane protein.

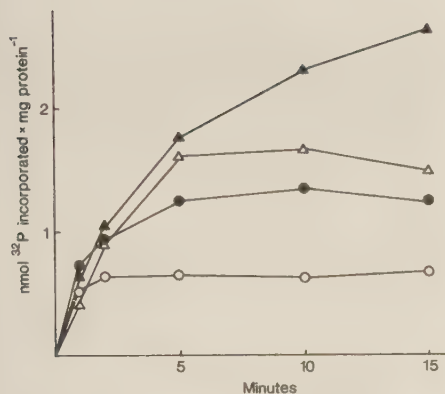


Fig. 2. Time course of endogenous incorporation of phosphate into plasma membranes. Incubations were performed as described under Experimental, and the incorporation of phosphate into trichloroacetic acid-sodium tungstate precipitable material was determined. Symbols represent the same amounts of plasma membranes and sodium azide as in Fig. 1.

The time courses of plasma membrane protein kinase activity obtained with various amounts of membrane protein and 2 mM ATP are presented in Fig. 1. The effect of the ATPase inhibitor sodium azide was also tested. The rate of incorporation was linear for 2 min at a low membrane protein concentration ($3 \mu\text{g}$ per $50 \mu\text{l}$ incubation medium) and then decreased slowly. At $20 \mu\text{g}$ the incorporation levelled off already after 2 min. This was probably due to exhaustion of ATP caused primarily by membrane ATPase, since the reaction proceeded for a longer time in the presence of azide. In comparison, when the incubation conditions used earlier for microsomes and mitochondria^{3,9} were tested with plasma membranes the incorporation ceased within 20 s.

The endogenous incorporation of phosphate into plasma membranes followed similar time courses as the protein kinase activity (Fig. 2), although the initial reaction rate was 10 times lower than with exogenous substrate. At the low membrane concentration, maximum incorporation was reached after 5 min with 1.6 nmol of phosphate incorporated per mg of membrane protein. Further incorporation was obtained in the presence of azide. Cyclic

Table 1. Extraction of plasma membrane components with KCl and Triton X-100. Purified plasma membranes were treated with KCl or KCl and Triton X-100 as described in the experimental section. Analyses were performed on extracts and unextracted material (pellets) and the distribution of recovered activities was calculated.

Treatment	Protein kinase activity (nmol min^{-1})		Endogenous incorporation of phosphate (nmol)		Protein (mg)		5'-Nucleotidase ($\mu\text{mol min}^{-1}$)	
	Extract	Pellet	Extract	Pellet	Extract	Pellet	Extract	Pellet
Membrane suspension	3.63		1.55		2.10		0.97	
	0.23	5.39	0.11	1.29	0.20	1.13	0	0.39
	0.81	4.33	0.06	0.92	0.22	1.11	0.004	0.35
	1.92	1.30	0.66	0.29	1.22	0.42	0.065	0.31
0.3 M KCl + 1 % Triton X-100	2.50	0.35	0.54	0.27	1.25	0.33	0.46	0.19
1.0 M KCl + 1 % Triton X-100								

AMP did not stimulate the phosphorylation of either endogenous or exogenous substrates.

Extraction of protein kinase from plasma membranes. To examine how tightly the protein kinase activity was attached to the membranes, extraction experiments were performed. Isolated plasma membranes were treated with various KCl and Triton X-100 concentrations and the distribution of protein kinase, endogenous phosphorylation, protein and the plasma membrane marker enzyme 5'-nucleotidase between solubilised and residual material was measured (Table 1). No 5'-nucleotidase activity and only a small fraction of protein kinase, endogenously phosphorylated material and protein was released from the plasma membranes on treatment with 0.3 M or 1 M KCl. However, a major part of protein kinase activity, endogenously phosphorylated material and protein was extracted in the presence of both salt and Triton X-100. The bulk of the protein appeared to be extracted under less drastic conditions than the protein kinase, since the protein was released at lower concentrations of Triton X-100 than most of the protein kinase activity (not shown). Most of the 5'-nucleotidase activity remained in the particulate fraction at 0.3 M KCl and 1 % Triton X-100, but was released when the salt concentration was increased to 1 M in presence of the detergent.

The protein kinase activity of plasma membranes increased by approximately 50 % on treatment

with KCl. The recovery of protein kinase after detergent treatment was lower, however, than the activity present in the parent membrane suspension. This is in contrast to what we have found earlier in microsomes and mitochondria where the protein kinase activity was latent in the membrane and increased several-fold in the presence of Triton X-100.^{3,9}

DEAE-cellulose chromatography. Plasma membranes were treated with 1 % Triton X-100 and 1 M KCl, and the solubilised material was subjected to DEAE-cellulose chromatography. Two peaks (PM I and PM II) with protein kinase activity were resolved (Fig. 3). When eluted from the column none of these peaks was stimulated by cyclic AMP. The two peaks eluted at the same salt concentrations (0.03 and 0.20 M, respectively) as two (M I and M III) of the three protein kinase peaks from rat liver microsomes³ and rat liver mitochondria.⁹ Two protein kinases that elute under these conditions have also been found in rat liver cytosol, which, in addition, contains two other enzyme peaks.³ Occasionally a third plasma membrane protein kinase fraction was observed which eluted at the same KCl concentration (0.14 M) as the M II fraction of microsomes and mitochondria.

Characterisation of the protein kinase fractions. Analyses of the protein kinase peaks from microsomes and the cytosol³ have shown that the first eluting peak is the catalytic subunit of the cyclic

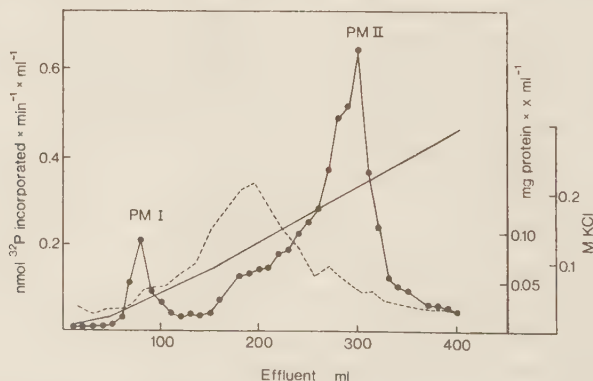


Fig. 3. DEAE-cellulose chromatography of plasma membrane protein kinase activity. Conditions used were as described in the experimental section. Protein kinase activity (●) was determined with protamine as substrate. ---, protein concentration measured according to Lowry; —, KCl gradient. The applied amount of protein was 15 mg.

Table 2. Substrate specificity of protein kinases and activity in presence of protein kinase inhibitor. Protein kinases were isolated from plasma membranes and cytosol by DEAE-cellulose chromatography. Their substrate specificities were determined in presence of 5 μ M cyclic AMP. The designations of the cytosolic protein kinases are the same as in Ref. 3. Activities are expressed relative to the activity towards protamine in presence of 0.3 M NaCl. The activity with protein kinase inhibitor was determined under conditions of maximum inhibition with protamine as substrate.

Substrate	Plasma membrane protein kinase		Cytosolic protein kinase			
	PM I	PM II	CI	C IIa	C IIb	C III
Protamine + NaCl	100	100	100	100	100	100
Protamine - NaCl	42	106	41	41	90	77
Histone mixture	18	9	7	9	4	12
Activity with protein kinase inhibitor	14	70	24	27	97	52

AMP dependent protein kinases, the second peak is a cyclic AMP independent enzyme while the third peak is the cyclic AMP dependent type II²⁷ protein kinase. Isoelectric focusing of PM I showed that this peak had the same isoelectric point ($pI=8.1$) as the catalytic subunit isolated from the cytosol.³ Although the PM II fraction was unstable on isoelectric focusing (the recovery was 2 % compared to 25–40 % obtained for other protein kinases examined in this manner) two protein kinase peaks could be observed with the isoelectric points 5.5 and 4.9. These correspond to the pI values we obtained earlier with the cyclic AMP-independent protein kinase and the cyclic AMP-dependent type II enzyme, respectively.³

The PM I and PM II protein kinase fractions were characterised further by their substrate specificity (of particular interest was the influence of NaCl on the phosphorylation of protamine) and the effect of a protein kinase inhibitor isolated from rabbit skeletal muscle (Table 2). Both substrate specificity and maximum inhibition by the protein kinase inhibitor were similar for PM I and the cytosolic protein kinase fraction C I (for designations of cytosolic kinases, see Ref. 3), providing further evidence that PM I is the catalytic subunit of cyclic AMP-dependent protein kinases. The activity of the PM II fraction decreased 30 % in the presence of inhibitor, and the rate of protamine phosphorylation by this enzyme was independent of the concentration of NaCl. In comparison, the decrease in activity of the cyclic AMP-dependent cytosolic protein kinases C IIa and C III due to the presence of inhibitor was 73 and 48 %, respectively, while the cyclic AMP-independent enzyme C IIb

was unaffected. The lower inhibition of fraction C III compared to C IIa was probably due to an incomplete dissociation of the holoenzyme under the experimental conditions used. The phosphorylation of protamine catalysed by fraction C IIb was also unaffected by 0.3 M NaCl, while the usual stimulation by salt was observed for the cyclic AMP-dependent enzyme fractions. The rate of histone phosphorylation was similar for the different enzyme fractions.

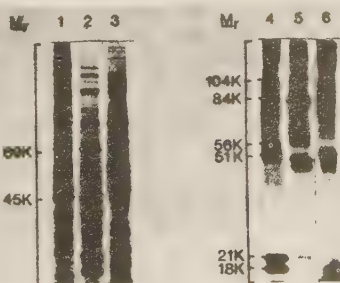


Fig. 4. Protein and phosphoprotein patterns of plasma membranes (lane 1 and 4), cytosol (2 and 5) and smooth microsomes (3 and 6) from rat liver. 1–3, protein patterns; 4–6, phosphoprotein patterns obtained by autoradiography. Standard proteins are indicated to the left of lane 1 and the apparent M_r of phosphorylated components to the left of lane 4. The protein patterns are from a different run than the phosphoprotein patterns. Smooth microsomes and cytosol were prepared as previously described.³

Phosphoprotein and protein patterns. To examine phosphoprotein and protein patterns, plasma membranes were phosphorylated endogenously using [γ - 32 P]ATP followed by SDS-polyacrylamide gel electrophoresis and autoradiography. For a comparison we also examined smooth endoplasmic reticulum and cytosol from rat liver in the same way. The protein patterns of plasma membranes, smooth microsomes and cytosol were distinctly different (Fig. 4), indicating that plasma membranes were not seriously contaminated by the latter two fractions. The phosphoprotein patterns were also different. Five prominent phosphorylated components (labelled 84K, 56K, 51K, 21K, and 18K in the figure after their apparent M_r) and additional minor ones were resolved from plasma membranes. Three of the major components seemed to be unique to plasma membranes while one (21K) had the same mobility as a phosphorylated component in the cytosol and another (18K) the same as one of the microsomal components. The 18K and 21K components remain unidentified so far, and it is not known whether they represent proteins present in more than one subcellular fraction, or whether they fortuitously have the same mobility as unrelated phosphorylated proteins in microsomes or the cytosol.

The phosphoprotein pattern of plasma membranes was also examined after various incubation times (not shown). The strength of all radioactive bands increased similarly when the incorporation of phosphate increased indicating a simultaneous phosphorylation of the components under the experimental conditions used.

The phosphoprotein patterns obtained after different extractions are shown in Fig. 5. Four prominent phosphorylated components (59K, 49K, 41K, 39K) were seen in the 1M KCl-extract. Of these the 59K, 49K and 39K components were not seen in the parent membrane, while the 41K component was a minor phosphorylated band in the native membrane which became heavily phosphorylated after extraction. Additional phosphoprotein bands (104K and 51K) were seen in the KCl and Triton X-100 extract. Interestingly, the 56K, 51K (in part) and 21K components remained in the particulate fraction after this drastic treatment which removed 80% of the membrane proteins, while the 18K component was found neither in the extract nor in the pellet.

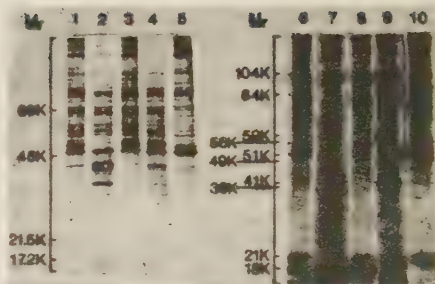


Fig. 5. Protein and phosphoprotein patterns of plasma membranes after various extractions. Extractions were performed with 1M KCl or 1% Triton X-100 and 1M KCl as described in the experimental section. Extracts and residues were phosphorylated endogenously before electrophoresis and autoradiography. 1-5 are protein patterns and 6-10 are phosphoprotein patterns in the order plasma membranes, KCl extract, residue after KCl extraction, Triton X-100 + KCl extract, residue after Triton X-100 + KCl extraction. Standard proteins are indicated to the left of lane 1 and the apparent M_r of phosphorylated components to the left of lane 6.

DISCUSSION

Several pieces of evidence indicate that the isolated protein kinases and phosphoproteins are harboured in the plasma membrane rather than representing contamination from other subcellular fractions. First, plasma membranes were substantially enriched during the preparation compared to other subcellular fractions (for instance, they were enriched 35-fold as compared to microsomal membranes). Second, both protein and phosphoprotein patterns were unique to the plasma membrane fraction. Third, no latency of protein kinase activity was observed when plasma membrane were treated with Triton X-100. In comparison, there is a several-fold increase in the kinase activity upon treatment of microsomes,² mitochondria⁹ or Golgi membranes (unpublished) with the detergent. Furthermore, the protein kinases appeared to be tightly bound to the membrane, since a rather harsh treatment with detergent and a high salt concentration were required to solubilise the enzyme activity.

Cyclic AMP did not stimulate the membrane-associated or solubilised kinase when analysed with

either endogenous or exogenous substrates, contrary to what has been found for plasma membrane protein kinases from other kinds of cells.^{28,29} We observed, however, that cyclic AMP-binding material is present in liver plasma membranes (approximately 2 pmol cyclic AMP bound per mg of membrane protein). This material has not been further characterised and it is unclear whether it represents any of the regulatory subunits of cyclic AMP-dependent protein kinases or other cyclic AMP-binding components.

The specific activity of the protein kinase of plasma membranes was several-fold higher than in microsomes³ or mitochondria.⁹ However, the enzyme activity of plasma membranes did not show any latency on detergent treatment in contrast to the activity in microsomes or the mitoplast fraction of mitochondria. The lack of latency indicates that the substrate (protamine) is freely accessible to the protein kinase. Since we have found in preliminary experiments that only a few proteins in isolated plasma membranes become significantly labelled by diazotised iodosulfanilic acid, all membranes probably expose the same surface to the surrounding medium. The protein kinase may therefore be attached to only one plasma membrane surface in intact cells. This is likely to be the outer surface.^{30,31} It has been reported that protein kinases are exposed on the outer surface of various kinds of cells including adipocytes³² and Ehrlich ascites tumour cells.³³

The protein kinase of plasma membranes was resolved into two enzymically active fractions on DEAE-cellulose chromatography. The first eluting fraction (PM I) is likely to represent the catalytic subunit of cyclic AMP-dependent protein kinases based on its isoelectric point, its substrate specificity and its inhibition by the protein kinase inhibitor. We have found earlier that the catalytic subunit apart from being present in the cytosol is also present in small amounts in rat liver microsomes³ and mitochondria.⁹ The subunit therefore seems to be widely distributed in the cell. The possibility that it is a contaminant from the cytosol appears less likely since a cytosolic enzyme as lactate dehydrogenase was present in a very low concentration in purified plasma membranes and since drastic conditions were required to solubilise protein kinase from the membrane.

The identity of the second kinase fraction (PM II) is less clear. It eluted from DEAE-cellulose at the same KCl concentration as the cyclic AMP-

dependent type II protein kinase which also seems to be present in microsomes and mitochondria.^{3,9} The PM II fraction was partly inhibited by the protein kinase inhibitor, but contrary to what is expected for cyclic AMP-dependent protein kinases,³ NaCl did not activate the phosphorylation of protamine by this fraction. Furthermore, the enzyme activity was unaffected by cyclic AMP when eluted from the column. However, a stimulation by this nucleotide is not necessarily expected with such a dilute enzyme as in this case (*cf.* Ref. 9). Due to the lability of the enzyme we have not yet been able to perform detailed studies of its properties.

Only a few proteins became heavily phosphorylated on endogenous phosphorylation of isolated plasma membranes. Most of these phosphoproteins were tightly bound to the particulate material since they were not released by treatment of the membrane with detergent and high KCl concentrations. Some proteins which were not phosphorylated significantly in the isolated membrane were amenable to phosphorylation after treatment with KCl and/or detergent. It is possible therefore that there are restrictions in enzyme-substrate interactions in the isolated membrane, either due to different transverse locations of enzymes and substrates, or to restrictions in their lateral mobility. If some protein substrates are located on the inner surface of intact cells while protein kinases are located on the outside, these substrates may be available for phosphorylation only by cytosolic protein kinases. We are presently studying the transverse location of protein kinases and their substrates in plasma membranes to resolve these questions.

As to the biochemical significance of protein phosphorylation in rat liver plasma membranes, one can only speculate as long as the phosphoproteins remain unidentified. Since protein phosphorylation in general seems to be connected with various regulatory functions in the cell, it is likely that the phosphorylation of proteins in plasma membranes also is a regulatory device. Thus, it has been suggested that the state of protein phosphorylation in erythrocyte membranes may regulate the membrane structure,³⁴ and there is indirect evidence for a correlation between protein phosphorylation and the movement of Na⁺ ions through the erythrocyte membrane.³⁵ There is also a recent report³⁶ on protein kinase-catalysed phosphorylation of the Na⁺, K⁺-ATPase of kidney plasma membranes.

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CYCLIC AMP-DEPENDENT PROTEIN PHOSPHORYLATION ON THE SURFACE OF RAT HEPATOCYTES

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1. Introduction

Proteins of isolated plasma membranes from rat liver cells can be phosphorylated either by endogenous [1–3] or exogenously added [4] protein kinases. Protein phosphorylation in plasma membranes presumably serves as a general regulatory mechanism as is the case in other subcellular fractions. An example is the phosphorylation and thereby activation of a cyclic nucleotide phosphodiesterase associated as a peripheral protein with rat liver plasma membranes [5]. The phosphorylation of other, as yet unidentified, plasma membrane proteins appears to be modulated by insulin [3].

No studies have been performed so far on the phosphorylation of proteins on the external surface of isolated rat hepatocytes. The phosphorylation of surface proteins in other kinds of cells has been described, however [6–8]. An examination of surface protein phosphorylation may yield information about regulatory mechanisms on the plasma membrane level. Here we report on cyclic AMP-dependent and independent phosphorylation of proteins on the outer surface of rat hepatocytes.

2. Experimental

Hepatocytes were prepared [9,10] from male Sprague-Dawley rats weighing 200–300 g. Bicarbonate-free Hanks' medium [11], buffered to pH 7.4 with 20 mM HEPES, also containing 0.05% collagenase, 0.1% hyaluronidase and 0.2% bovine serum albumin (all from Sigma) was used for the perfusion. The viability of the cells, measured as exclusion of 0.1% trypan blue, was above 90%.

In control experiments to examine the influence of leakage of protein kinase and cyclic adenosine 3', 5'-monophosphate (cyclic AMP) binding proteins on hepatocyte surface phosphorylation 1 g of hydroxyapatite-agarose gel (HA-Ultrogel, LKB) was added per g liver, when the perfused and collagenase-treated liver was dispersed in medium. The gel was present during the entire handling of the cells before phosphorylation analyses. The recovery of cells dropped to approx. 50% by this treatment due to adsorption of cells to the gel, but the viability of the remaining cells increased to above 95%.

Protein kinase analyses were performed (usually in triplicate) at 37°C in Ca^{2+} -free Hanks' medium buffered with 20 mM HEPES, pH 7.4. 1 ml of incubation mixture contained 2 μmol [$\gamma\text{-}^{32}\text{P}$]ATP (100–150 dpm/pmol; prepared as in [12]), 3 mg histones (Sigma, type II A), $10\text{--}15 \times 10^6$ hepatocytes, and (when indicated) 5 nmol cyclic AMP. At indicated times 50 μl of the mixture was spotted on a Whatman 3 MM filter paper disc, which was washed and counted as in [13]. Protein phosphatase activity was followed after the addition of hexokinase (Sigma, 180 U/ml) to remove unreacted ATP (glucose is present in the incubation medium). In some experiments 360 $\mu\text{g/ml}$ rabbit muscle protein kinase inhibitor (Sigma) was added.

Endogenous phosphorylation of hepatocytes was performed as above but omitting histones. The filter paper discs were immersed immediately in 10% trichloroacetic acid. Before counting they were washed in the following way to remove unreacted ATP and radioactively labelled phospholipids (particularly phosphoinositides, cf. [14]): twice for 10 min in 10% trichloroacetic acid and 1 M H_3PO_4 , boiled once for 15 min in 5% trichloroacetic acid, washed once each for 5 min in 10% trichloroacetic acid and 1 M H_3PO_4 ,

ethanol, ethanol/ether (1:1, by vol.), chloroform/methanol 12 M HCl (200:100:1, by vol.), and ether. The discs were dried and counted in 0.4% Omnifluor (New England Nuclear) in toluene in a liquid scintillation counter.

Samples for electrophoresis were incubated for 5 min in 50 μ l medium also containing 0.1 μ mol [γ - 32 P]ATP (800–1000 dpm/pmol), 200 000 hepatocytes, and 0.25 nmol cyclic AMP (when indicated). SDS-polyacrylamide gel electrophoresis and autoradiography of the gels were as in [15,16].

3. Results

3.1. Phosphorylation of exogenous substrate

To test whether hepatocytes possess protein kinase activity on their external surface, isolated cells were incubated with [γ - 32 P]ATP and histones as exogenous substrate (fig.1). There was an incorporation of radioactively labelled phosphate, and cyclic AMP stimulated the reaction by approx. 50%. The cyclic AMP-dependent incorporation was partly inhibited by rabbit muscle protein kinase inhibitor [17], which acts specifically on cyclic AMP-dependent protein kinases. When hexokinase was added to remove unreacted ATP, a time-dependent decrease of bound phosphate was observed (—). This indicates the presence of phosphoprotein phosphatase activity as well on the cell surface.

The rate of phosphate incorporation was fast initially, and then slower and close to linear for a few

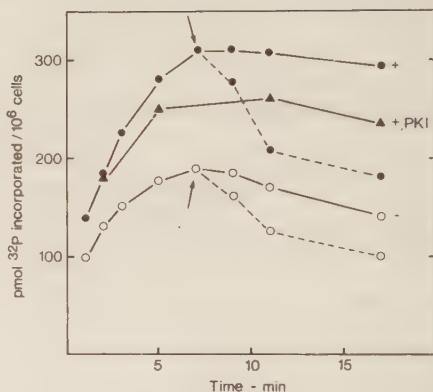


Fig.1. Phosphorylation and dephosphorylation of histones by hepatocytes. Analyses were performed as in section 2 in presence (+) or absence (—) of cyclic AMP. Arrows indicate addition of hexokinase to follow the dephosphorylation (---). PKI, protein kinase inhibitor.

minutes. The rate of incorporation was then approx. 40 and 25 pmol of phosphate/ 10^6 cells per min in presence and absence of cyclic AMP, respectively. The rate of dephosphorylation was also higher in presence of cyclic AMP, presumably due to a higher concentration of phosphate groups on the substrate in this case as compared to incubations without cyclic AMP.

The possibility that the protein kinase activity was due to an association of intracellular enzymes released

Table 1
Effect of adsorption of released protein kinase on hepatocyte surface phosphorylation

Treatment during cell preparation	Protein kinase in cell washes (nmol/min per g liver)		Cyclic AMP binding in cell washes (pmol/g liver)	Hepatocyte protein kinase (pmol/min per 10^6 cells)		Endogenous phosphorylation (pmol/min per 10^6 cells)	
	— ^a	+ ^a		—	+	—	+
Untreated	4.36	6.22	6.23	23.1	30.8	2.87	3.62
Hydroxyapatite agarose gel	1.12	1.21	1.34	36.0	44.1	3.52	3.95

After dispersion of the collagenase-treated liver in the absence or presence of hydroxyapatite the cells were incubated for 15 min at 37°C and washed twice as in [10]. The combined supernatants were analyzed for protein kinase (see section 2) and cyclic AMP binding (by a slight modification of [20]), and the cells for protein kinase and endogenous phosphorylation

^a — and +, in the absence or presence of cyclic amp, respectively

from leaking or broken cells during the preparation was considered. Approx. 80% of the released protein kinase and cyclic AMP-binding proteins were adsorbed by including hydroxyapatite in the cell preparation medium (table 1). The concentration of protein kinase on the hepatocyte surface did not decrease by this treatment (it rather increased in this particular, but not in other, control experiments), which would have been expected if released enzyme components associate with the cell surface. The hepatocytes could also be incubated with liver cytosol without an increase in the surface protein kinase activity (not shown).

3.2. Endogenous phosphorylation

The incorporation of phosphate into endogenous substrates was examined next (fig.2). The rate of this reaction was approx. 15 times lower than with histones as substrates (3 and 2 pmol incorporated/ 10^6 cells per min in presence and absence of cyclic AMP, respectively). A dephosphorylation of endogenously phosphorylated material occurred after addition of hexokinase to remove unreacted ATP. The rate and extent of this dephosphorylation was less than that observed with exogenous substrate. The endogenous phosphorylation was not affected when cells were prepared in the presence of hydroxyapatite to adsorb material released from leaking or broken cells (table 1).

The protein kinase inhibitor from rabbit skeletal muscle inhibited endogenous phosphate incorporation (fig.2). The rate of incorporation in presence of both inhibitor and cyclic AMP was similar to that obtained

Table 2
Subcellular location of phosphorylated components

Preparation	Total incorporation	Super-natant	Pellet	% recovery
Intact cells	21.9	0.1	9.9	46
Homogenized cells	73.2	6.8	35.6	58

Hepatocytes or homogenized cells (30 strokes of a tight-fitting Dounce homogenizer) were incubated with [γ - 32 P]ATP for 5 min at 37°C. A 10-fold excess of unlabelled ATP and 0.05 M sodium fluoride were added at 0°C to stop further labelling and to inhibit phosphatase activity. The cells were homogenized as above and centrifuged at $110\,000 \times g$ for 90 min. Samples from supernatants and pellets were spotted on filter paper discs and washed as for endogenous phosphorylation before counting. The incorporation is given as pmol/ 10^6 cells

without these additions. The inhibitor did not affect the cyclic AMP-independent incorporation appreciably (not shown).

To verify that the endogenous phosphorylation occurred on the hepatocyte surface, and was not due to leakage of radioactively labelled ATP into the cells or of intracellular material to the medium, we examined the incorporation of phosphate into soluble and particulate fractions of hepatocytes (table 2). The radioactivity was recovered almost exclusively in the particulate fraction after incubation of intact cells with radioactively labelled ATP. If, however, the cells were homogenized before the incubation, an appreciable incorporation was into the soluble fraction, i.e. cytosolic material. The incorporation into particulate material was also higher in homogenized cells, presumably due to the phosphorylation of components in the exposed intracellular membranes. These findings strongly indicate that the incorporation into intact cells was localized to the cell surface.

3.3. Phosphoprotein patterns

Phosphoprotein patterns were examined by SDS-polyacrylamide gel electrophoresis and autoradiography after incubation of hepatocytes with radioactively labelled ATP (fig.3). A few proteins became labelled, and the incorporation into 5 of these (indicated by dots) was dependent on cyclic AMP. Presumably the incorporation was into surface proteins, since the pattern did not change by including hydroxy-

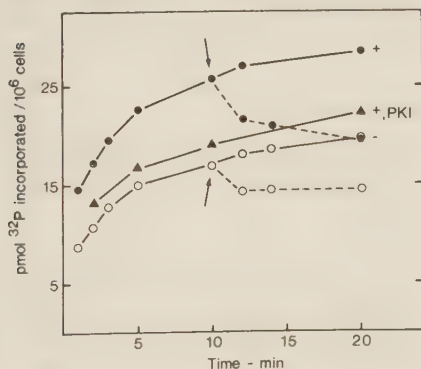


Fig.2. Endogenous phosphorylation and dephosphorylation of hepatocyte surface proteins. See legend to fig.1.

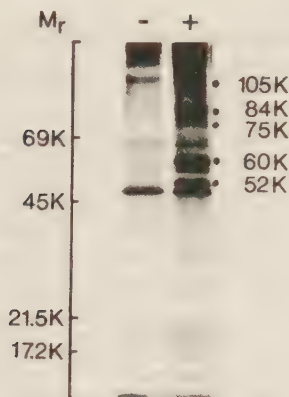


Fig. 3. Phosphoprotein patterns. Hepatocytes were incubated with [γ - 32 P]ATP in presence (+) or absence (-) of cyclic AMP. Proteins were separated by SDS-polyacrylamide gel electrophoresis and autoradiographed. Dots indicate components with a cyclic AMP-stimulated phosphorylation. The M_r of reference proteins is indicated to the left.

apatite in the cell preparation medium, and since no [32 P]phosphate was found in the soluble fraction after homogenization of the cells (see above). We have also found in other experiments that the phosphoprotein patterns obtained with homogenized cells or with rat liver cytosol are quite different from the patterns presented here.

4. Discussion

The results provide evidence for a surface location of protein kinase, phosphoprotein phosphatase, and substrates for these enzymes in isolated rat liver hepatocytes. At least a part of the exposed protein kinase activity is cyclic AMP dependent. The evidence for a surface location is based on the phosphorylation/dephosphorylation of histones (a substrate which presumably does not enter intact cells) and the incorporation of phosphate into particulate, but not into soluble, material in the cell. In addition, there was no lag phase in the phosphorylation of endogenous proteins. This would have been expected if labelled ATP or phosphate had to enter the cells before phosphorylation, since these would have to equilibrate with

intracellular pools to yield a maximum rate of incorporation. Thus, a lag phase of at least 10 min is seen in the phosphorylation of proteins when hepatocytes are incubated with [32 P]phosphate (unpublished).

The possibility that intracellular enzymes catalyzed the cell surface phosphorylation after leakage to the medium and association with the plasma membrane appears less likely, since 80% of the protein kinase and cyclic AMP-binding proteins set free during the cell preparation could be trapped without decreasing the protein kinase activity of the cells. The possible leakage of enzymes during collagenase treatment could not be checked, however. Since the liver was continuously perfused during this stage released enzymes were likely to be carried away before they could associate extensively with the cell surface. Cyclic AMP-dependent protein kinases at low concentrations do not bind extensively to microsomal membranes [18], and we did not observe any increase in the protein kinase activity of hepatocytes after incubation of the cells with liver cytosol. The leakage of protein kinase from the cells during analysis was also low.

The functional importance of a location of cyclic AMP-dependent protein kinase and substrates for this enzyme on the hepatocyte surface is a matter for speculation. It is in line, however, with the recent finding [19], that a fraction of the cellular cyclic AMP synthesis may take place on the outer surface of hepatocytes. Since a surface location of cyclic AMP-dependent protein kinase activity has been reported for several kinds of cells [7,8], this might be a property of most cells. It is of interest now to identify the phosphoproteins located on the hepatocyte surface, and to examine the function of these phosphorylations.

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